

LAW OFFICES

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October 16, 2000

BOX PCT

Assistant Commissioner
for Patents
Washington, D.C. 20231

PCT/AU99/00306 - filed
April 23, 1999

Re: Application of Susan J. CLARK,
Douglas S. MILLAR and Peter L. MOLLOY
entitled "ASSAY FOR METHYLATION IN
THE GST-Pi GENE"
Our Ref: Q-61152

Dear Sir:

The following documents and fees are submitted herewith in connection with the above application for the purpose of entering the National stage under 35 U.S.C. § 371 and in accordance with Chapter II of the Patent Cooperation Treaty:

- ☐ an executed Declaration and Power of Attorney.
- ☒ the International Application (in English).
- ☒ 17 sheets of drawings.
- ☒ Substitute pages 5-11 and 62-72.
- ☐ an English translation of Article 19 claim amendments.
- ☒ an International Preliminary Examination Report.
- ☒ Statement in Support of Submission (along with substitute Sequence Listing and DOS version diskette containing the same).
- ☐ an executed Assignment and PTO 1595 form.
- ☒ a Form PTO-1449 listing the ISR references and a complete copy of each reference.
- ☐ Written Opinion.
- ☒ a Preliminary Amendment.

09/673448

532 Rec'd PCT/PTC 16 OCT 2000

SUGHRUE, MION, ZINN, MACPEAK & SEAS, PLLC

Assistant Commissioner
of PatentOctober 16, 2000
Page 2

The Declaration and Power of Attorney and Assignment will be submitted at a later date.

Priority is claimed from April 23, 1998, based on Australian Application No. PP 3129.

The Government filing fee is calculated as follows:

Total claims	<u>50</u> - <u>20</u> = <u>30</u>	x	\$18.00	=	<u>\$540.00</u>
Independent					
claims	<u>4</u> - <u>3</u> = <u>1</u>	x	\$80.00	=	<u>\$80.00</u>
Base Fee					<u>\$1000.00</u>
Multiple Dependent Claim Fee					<u>\$0.00</u>

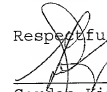
TOTAL FEE\$1620.00

A check for the statutory filing fee of \$1620.00 will be submitted shortly.

The Assistant Commissioner is hereby directed and authorized to charge or credit any difference or overpayment to Deposit Account No. 19-4880.

The Assistant Commissioner is also hereby authorized to charge any fees under 37 C.F.R. §§ 1.16, 1.17 and 1.492 which may be required during the entire pendency of the application to Deposit Account No. 19-4880. A duplicate copy of this transmittal letter is attached.

Respectfully submitted,


 Gordon Kit

Registration No. 30,764

**SUGHRUE, MION, ZINN,
MACPEAK & SEAS, PLLC**

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Washington, D.C. 20037-3213
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

Date: October 16, 2000

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Susan J. CLARK et al

Appln. No.: 09/673,448

Filed: October 16, 2000

For: ASSAY FOR METHYLATION IN THE GST-Pi GENE



Group Art Unit: 0000

Examiner: Unknown

**STATEMENT IN SUPPORT OF SUBMISSION
IN ACCORDANCE WITH 37 C.F.R. § 1.821**

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:


The following statement is provided to meet the requirements of 37 C.F.R. § 1.821.

I hereby state that the content of the computer readable copy (PatentIn Version 3.0) of the third substitute Sequence Listing (attached hereto) submitted in accordance with 37 C.F.R. §§ 1.821(c) and (e), respectively, is the same as the third substitute Sequence Listing submitted herewith.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge and that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

7/12/01

Date


Gordon Kit

10 MAY 2001

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Susan J. CLARK et al

Appln. No.: 09/673,448

Group Art Unit: 0000

Filed: October 16, 2000

Examiner: Unknown

For: ASSAY FOR METHYLATION IN THE GST-PI GENE

STATEMENT IN SUPPORT OF SUBMISSION
IN ACCORDANCE WITH 37 C.F.R. § 1.821

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

The following statement is provided to meet the requirements of 37 C.F.R. § 1.821.


I hereby state that the content of the computer readable copy (PatentIn Version 3.0) of the substitute Sequence Listing submitted in accordance with 37 C.F.R. §§ 1.821(c) and (e), respectively, is the same as the substitute Sequence Listing filed simultaneously herewith.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge and that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that

STATEMENT IN SUPPORT OF SUBMISSION
U.S. Appln. No. 09/673,448

such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

5/15/01
Date


Gordon Kit

09/673448

532 Rec'd PCT/PTC 16 OCT 2000

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

SUSAN J. CLARK et al

CHAPT II filing

Appln. No.: of PCT/AU99/00306

Group Art Unit: 0000

Filed: October 16, 2000

Examiner: Unknown

For: ASSAY FOR METHYLATION IN THE GST-Pi GENE

STATEMENT IN SUPPORT OF SUBMISSION
IN ACCORDANCE WITH 37 C.F.R. § 1.821

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

The following statement is provided to meet the requirements of 37 C.F.R. § 1.821.

I hereby state that the content of the computer readable copy (PatentIn Version 2.1) of the substitute Sequence Listing submitted in accordance with 37 C.F.R. §§ 1.821(c) and (e), respectively, is the same as the substitute Sequence Listing filed simultaneously herewith.


I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge and that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that

STATEMENT IN SUPPORT OF SUBMISSION
CHAPTER II FILING OF PCT/AU99/00306

such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

10/6/00

Date



Gordon Kit

09/673448

532 Rec'd PCT/PTC 16 OCT 2000

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

SUSAN J. CLARK et al

CHAPT II filing

Appln. No.: of PCT/AU99/00306

Group Art Unit: 0000

Filed: October 16, 2000

Examiner: Unknown

For: ASSAY FOR METHYLATION IN THE GST-Pi GENE

PRELIMINARY AMENDMENT

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

Prior to examining the above-identified application, please
amend the application as follows.

IN THE SPECIFICATION:

Page 1, before line 3, insert

-- This application is a 371 of PCT/AU99/00306 filed
April 23, 1999. --

Pages 5-11, delete in their entirety, and insert therefor
substitute pages 5-11 filed simultaneously herewith.

Substitute pages 62-72 (filed simultaneously herewith),
please renumber as pages 45-55, respectively.

IN THE CLAIMS:

Please amend the claims (substitute Claims 1-50, i.e.,
substitute pages 62-72, filed simultaneously herewith) as
follows:

PRELIMINARY AMENDMENT
CHAPTER II Filing
of PCT/AU99/00306

Claim 3, line 1, change "any one of the preceding claims"
to -- Claim 1 --.

Claim 14, line 1, change "any one of the preceding claims"
to -- Claim 1 --.

Claim 15, line 1, change "any one of the preceding claims"
to -- Claim 1 --.

Claim 22, line 1, change "any one of the preceding claims"
to -- Claim 1 --.

Claim 23, line 1, change "any one of claims 5 to 21" to
-- Claim 5 --.

Claim 37, line 1, delete "or 36".

Claim 40, line 1, change "any one of claims 35 to 39" to
-- Claim 35 --.

Claim 42, line 1, change "any one of claims 35 to 41" to
-- Claim 35 --.

Claim 44, line 1, change "any one of claims 35 to 43" to
-- Claim 35 --.

Claim 45, line 1, change "any one of claims 35 to 43" to
-- Claim 35 --.

IN THE SEQUENCE LISTING:

Pages 45-61 (Sequence Listing), please in its entirety.

IN THE ABSTRACT:

Please insert the Abstract attached hereto.

**PRELIMINARY AMENDMENT
CHAPTER II Filing
of PCT/AU99/00306**


REMARKS

The specification has been amended to insert formal matter, the claims have amended to delete their multiply dependency and the Abstract has been inserted in order to make the application consistent with U.S. patent practice. Hence, the amendment of the specification and claims and the addition of the Abstract does not constitute new matter.

The Examiner is requested to note that Applicants simultaneously file herewith a substitute Sequence Listing (which is now considered to be a separate document by the U.S. Patent and Trademark Office) in PatentIn Version 2.1. Hence, the present Sequence Listing in the above-identified application is not necessary.

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

Respectfully submitted,



Gordon Kit
Registration No. 30,764

**SUGHRUE, MION, ZINN,
MACPEAK & SEAS, PLLC**
2100 Pennsylvania Avenue, N.W.
Washington, D.C. 20037-3202
(202) 293-7060

Date: October 16, 2000

PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Susan J. CLARK et al

Appln. No.: 09/673,448

Group Art Unit: 0000

Filed: October 16, 2000

Examiner: Unknown

For: ASSAY FOR METHYLATION IN THE GST-Pi GENE

SECOND PRELIMINARY AMENDMENT

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

Further to the Preliminary Amendment of October 16, 2000,
and prior to examining the above-identified application, please
amend the application as follows.

IN THE SEQUENCE LISTING:

Please delete the present substitute Sequence Listing and
insert therefor the attached second substitute Sequence Listing.


REMARKS

Applicants simultaneously file herewith a second substitute
Sequence Listing (in order to correct the formatting errors as
noted in the Notification of Missing Requirements dated
April 19, 2001) in PatentIn Version 3.0. Hence, the submission of
the present second substitute Sequence Listing does not raise new
issues or constitute new matter.

SECOND PRELIMINARY AMENDMENT
U.S. Appln. No. 09/673,448

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

~~Respectfully~~ submitted,


Gordon Kitz

Registration No. 30,764

SUGHRUE, MION, ZINN,
MACPEAK & SEAS, PLLC
2100 Pennsylvania Avenue, N.W.
Washington, D.C. 20037-3202
(202) 293-7060

Date: May 10, 2001



PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Susan J. CLARK et al

Appln. No.: 09/673,448

Group Art Unit: 0000

Filed: October 16, 2000

Examiner: Unknown

For: ASSAY FOR METHYLATION IN THE GST-Pi GENE

THIRD PRELIMINARY AMENDMENT

Assistant Commissioner
of Patents
Washington, D.C. 20231

Sir:

Further to the Second Preliminary Amendment filed May 10, 2001, and the Preliminary Amendment of October 16, 2000, and prior to examining the above-identified application, please amend the application as follows.

IN THE SEQUENCE LISTING:

Please delete the present second substitute Sequence Listing and insert therefor the attached third substitute Sequence Listing.

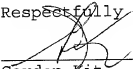
REMARKS

Applicants simultaneously file herewith a third substitute Sequence Listing (in order to correct the errors noted in the Raw Sequence Listing attached to the Notification to Comply with Requirements dated June 29, 2001) in PatentIn Version 3.0. Hence, the submission of the present third substitute Sequence Listing does not raise new issues or constitute new matter.

THIRD PRELIMINARY AMENDMENT
U.S. Appln. No. 09/673,448

The Examiner is invited to contact the undersigned at his Washington telephone number on any questions which might arise.

Respectfully submitted,


Gordon Kit

Registration No. 30,764

SUGHRUE, MION, ZINN,
MACPEAK & SEAS, PLLC

2100 Pennsylvania Avenue, N.W.
Washington, D.C. 20037-3202
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

Date: July 12, 2001

Sequence Listings:

Applicant: Commonwealth Scientific and Industrial Research
Organisation

Title: Diagnostic assay

Prior Application Number: PF3129

Prior Application Filing Date: 1998-04-23

Number of SEQ ID NOS: 59

Software: PatentIn Ver. 2.1

SEQ ID NO: 1

Length: 29

Type: DNA

Organism: Homo sapiens

Sequence: 1

cgcgagggttt tcgttgaggt ttcgtcgtc

29

SEQ ID NO: 2

Length: 25

Type: DNA

Organism: Homo sapiens

Sequence: 2

cgttattagt gagtacgcgc ggttc

25

SEQ ID NO: 3

Length: 24

Type: DNA

Organism: Homo sapiens

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Sequence: 3
yggtttttagg gaattttttt tcgc 24

SEQ ID NO: 4
Length: 28
Type: DNA
Organism: Homo sapiens

Sequence: 4
ygggyggtta gttggtggy tatatttc 28

SEQ ID NO: 5
Length: 29
Type: DNA
Organism: Homo sapiens

Sequence: 5
gggaattttt tttcgcgatg tttyggcgc 29

SEQ ID NO: 6
Length: 24
Type: DNA
Organism: Homo sapiens

Sequence: 6
tttttagggg gtttyggagcg tttc 24

SEQ ID NO: 7
Length: 19
Type: DNA
Organism: Homo sapiens

Sequence: 7
ggtaggttgy gtttatcgc 19

SEQ ID NO: 8

3/17

Length: 27

Type: DNA

Organism: Homo sapiens

Sequence: 8

aaaaattcra atctctccga ataaacg

27

SEQ ID NO: 9

Length: 27

Type: DNA

Organism: Homo sapiens

Sequence: 9

aaaaaccraa ataaaaacca cacgacg

27

SEQ ID NO: 10

Length: 25

Type: DNA

Organism: Homo sapiens

Sequence: 10

tcccatccct cccgaaacg ctccg

25

SEQ ID NO: 11

Length: 33

Type: DNA

Organism: Homo sapiens

Sequence: 11

gaacgcctcc gaaccccta aaaaccgcta acg

33

SEQ ID NO: 12

Length: 27

Type: DNA

Organism: Homo sapiens

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Sequence: 12
crrccctaaaa tccccraaat crccgcg 27

SEQ ID NO: 13
Length: 30
Type: DNA
Organism: Homo sapiens

Sequence: 13
accccracra ccctacacc ccraacgtcg 30

SEQ ID NO: 14
Length: 31
Type: DNA
Organism: Homo sapiens

Sequence: 14
ctcttctaaa aaatcccrxr aactcccgcc g 31

SEQ ID NO: 15
Length: 29
Type: DNA
Organism: Homo sapiens

Sequence: 15
aaaacrcctt aaaatccccg aaatcgccg 29

SEQ ID NO: 16
Length: 30
Type: DNA
Organism: Homo sapiens

Sequence: 16
aactcccrcc gaccccaacc ccgacgaccg 30

SEQ ID NO: 17

5/17

Length: 23

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial

Sequence:Oligonucleotide

which binds bisulfite-converted human GST-Pi gene

Sequence: 17

aaacctaataa aataaataaa caa

23

SEQ ID NO: 18

Length: 23

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial

Sequence:Oligonucleotide

which binds non-converted human GST-Pi gene

Sequence: 18

gggcctaggg agtaaacaga cag

23

SEQ ID NO: 19

Length: 25

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial

Sequence:Oligonucleotide

which binds human GST-Pi gene

Sequence: 19

6/17

cctttccctc tttcccarrrt cccca

25

SEQ ID NO: 20

Length: 25

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial

Sequence:Oligonucleotide

which binds bisulfite-converted human GST-Pi gene

Sequence: 20

tttggtattt tttttcgggt tttag

25

SEQ ID NO: 21

Length: 25

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial

Sequence:Oligonucleotide

which binds non-converted human GST-Pi gene

Sequence: 21

cttgcatcc tccccgggc tccag

25

SEQ ID NO: 22

Length: 26

Type: DNA

Organism: Artificial Sequence

Feature:

7/17

Other Information: Description of Artificial

Sequence: Oligonucleotide

which binds human GST-Pi gene

Sequence: 22

gggagggaag ggaggyaggg gytggg

26

SEQ ID NO: 23

Length: 31

Type: DNA

Organism: Homo sapiens

Sequence: 23

ttatgtaata aatttgata tttgtatat g

31

SEQ ID NO: 24

Length: 25

Type: DNA

Organism: Homo sapiens

Sequence: 24

tgtagattat ttaaggttag gagtt

25

SEQ ID NO: 25

Length: 27

Type: DNA

Organism: Homo sapiens

Sequence: 25

aaacctaataa aataaaCaata caacaaa

27

SEQ ID NO: 26

Length: 29

Type: DNA

Organism: Homo sapiens

8/17

Sequence: 26
aaaaaacctt tccctctttc ccaaatccc 29

SEQ ID NO: 27
Length: 27
Type: DNA
Organism: Homo sapiens

Sequence: 27
tttgttggtt gtttattttt taggttt 27

SEQ ID NO: 28
Length: 26
Type: DNA
Organism: Homo sapiens

Sequence: 28
gggatttggg aaagagggaag aggttt 26

SEQ ID NO: 29
Length: 24
Type: DNA
Organism: Homo sapiens

Sequence: 29
actaaaaact ctaaacccca tccc 24

SEQ ID NO: 30
Length: 24
Type: DNA
Organism: Homo sapiens

Sequence: 30
aacctaatac taccttaacc ccat 24

9/17

SEQ ID NO: 31
Length: 33
Type: DNA
Organism: Homo sapiens

Sequence: 31
aatcctcttc ctactatcta ttactccct aaa 33

SEQ ID NO: 32
Length: 29
Type: DNA
Organism: Homo sapiens

Sequence: 32
aaaacctaaa aaaaaaaaaa aaacttccc 29

SEQ ID NO: 33
Length: 29
Type: DNA
Organism: Homo sapiens

Sequence: 33
ttgggtttat gttgggagtt ttgagtttt 29

SEQ ID NO: 34
Length: 29
Type: DNA
Organism: Homo sapiens

Sequence: 34
ttttgtgggg agttgggggt tgatgttgt 29

SEQ ID NO: 35
Length: 29
Type: DNA
Organism: Homo sapiens

10/17

Sequence: 35
ggtttagagt ttttagtatg gggttaatt 29

SEQ ID NO: 36
Length: 20
Type: DNA
Organism: Homo sapiens

Sequence: 36
tagtattagg ttagggtttt 20

SEQ ID NO: 37
Length: 29
Type: DNA
Organism: Homo sapiens

Sequence: 37
aactctaacc ctaatctacc aacaacata 29

SEQ ID NO: 38
Length: 29
Type: DNA
Organism: Homo sapiens

Sequence: 38
caaaaaactt taaataaacc ctcctacca 29

SEQ ID NO: 39
Length: 32
Type: DNA
Organism: Homo sapiens

Sequence: 39
gttttggtggt taggtgtgtt ttttaggtgtt ag 32

11/17

SEQ ID NO: 40

Length: 30

Type: DNA

Organism: Homo sapiens

Sequence: 40

gttttgagta tttgttggtt ggtagttttt 30

SEQ ID NO: 41

Length: 30

Type: DNA

Organism: Homo sapiens

Sequence: 41

ttaatatataa taaaaaaaaat atatttaca 30

SEQ ID NO: 42

Length: 34

Type: DNA

Organism: Homo sapiens

Sequence: 42

caacccccaa taccacaacc taatacaaat actc 34

SEQ ID NO: 43

Length: 26

Type: DNA

Organism: Homo sapiens

Sequence: 43

ggttttagtt tttggttggt tggatg 26

SEQ ID NO: 44

Length: 26

Type: DNA

12/17

Organism: Homo sapiens

Sequence: 44

ttttttgtt tttagtatat gtgggg 26

SEQ ID NO: 45

Length: 30

Type: DNA

Organism: Homo sapiens

Sequence: 45

ataactaaaa aactattttc taatcctcta 30

SEQ ID NO: 46

Length: 29

Type: DNA

Organism: Homo sapiens

Sequence: 46

ccaaactaaa aactcaaaa aaccactaa 29

SEQ ID NO: 47

Length: 38

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial Sequence: M13-human
GST-Pi oligonucleotide

Sequence: 47

tgtaaaacga cggccagtgg gatttgggaa agagggaa 38

SEQ ID NO: 48

Length: 38

Type: DNA

13/17

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial Sequence: M13-human
GST-Pi oligonucleotide

Sequence: 48

tgtaaaacga cggccagttg ttgggagttt tgagtttt 38

SEQ ID NO: 49

Length: 31

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial Sequence: M13-human
GST-Pi oligonucleotide

Sequence: 49

tgtaaaacga cggccagtta gtattaggtt a 31

SEQ ID NO: 50

Length: 37

Type: DNA

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial Sequence: M13-human
GST-Pi oligonucleotide

Sequence: 50

tgtaaaacga cggccagtggt tttaggtatt tgtgtgtg 37

SEQ ID NO: 51

Length: 35

Type: DNA

14/17

Organism: Artificial Sequence

Feature:

Other Information: Description of Artificial Sequence: M13-human
GST-Pi oligonucleotide

Sequence: 51

tgtaaacga cgccagtggt ttttagtata tgtgg

35

SEQ ID NO: 52

Length: 499

Type: DNA

Organism: Homo sapiens

Sequence: 52

tgcagatcac ctaagggtcag gagttcgaga ccagcccgcc caacatgggtg aaaccccgtc 60
tctactaaaa atacaaaaat cagccagatg tggcacgcac ctataattcc acctactcgg 120
gaggctgaag cagaattgct tgaacccgag aggcggaggtg tgcagtgagc cgccgagatc 180
gcgccactgc actccagcct gggccacagc gtgagactac gtcataaaat aaaataaaat 240
aacacaaaat aaaataaaat aaaataaaat aaaataaaat aataaaaaaa aataaaaaaa 300
aataaaaaaa aataaaaaaa agcaatttcc ttctctctaa gcggcctcca cccctctccc 360
ctgccctgtg aagcgggtgt gcaagctccg ggatcgagc ggtcttaggg aatttccccc 420
cgcatgtcc cgccgcgcc gttcgtctgc cactctcgc tgcggtcctc ttctgctgtg 480
ctgtttactc cctagcccc

499

SEQ ID NO: 53

Length: 316

Type: DNA

Organism: Homo sapiens

Sequence: 53

gggacctggg aaagagggaa aggcttcccc ggccagctgc gcggcgactc cggggactcc 60
agggcgcccc tctgcggcgg acgcccgggg tgcagcggcc gccggggctg gggccggcgg 120
gagtcgcggg gacctccag aagagcggcc ggcgcgtga ctcagcactg gggcgagcgg 180
gggcgggacc acccttataa ggctcgagg cgcgaggcc ttcgctggag ttccgcggcc 240
cgactcttcg ccaccagtga gtacgcgcgg cccgcgtccc cggggatggg gctcagagct 300

15/17

cccagcatgg ggccaa

316

SEQ ID NO: 54

Length: 603

Type: DNA

Organism: Homo sapiens

Sequence: 54

cagcatcagg cccgggctcc cggcagggt cctcgccac ctcgagacc gggacgggg 60
cctaggggac ccaggacgtc ccagtgccg ttagcggtt tcagggggcc cggagcgct 120
cggggaggga tgggaccccg gggcgggga gggggggcag gctgcgtca ccgcgcttg 180
gcatectccc ccgggtccca gcaaaccttt cttgttcgc tgcagtgcg ccctacaccg 240
tgggtctatt ccaggttcca ggtaggagca tgtgtctggc agggaaggga ggcaggggct 300
ggggctgcag cccacagccc ctgcccacc cggagagatc cgaacccct taccctccg 360
tcgtgtggct ttaccctcg gctccttcc tgttcccgc ctctccgac atgctgtctc 420
ccgccccag tgtgtgtga aatcttcgga ggaacctgtt tacctgttcc ctccctgcac 480
tcctgacccc tccccgggtt gctgcaggc ggagtcggcc cgggtccccc atctcgtact 540
tctccctccc cgcaggccgc tgcgcggccc tgcgcattgt gctggcagat caggggccaga 600
gct 603

SEQ ID NO: 55

Length: 266

Type: DNA

Organism: Homo sapiens

Sequence: 55

gctctgagca cctgctgtgt ggcagtctct catccttcca cgcacatcct cttccccctc 60
tcccagctg gggctcacag acagccccct ggttgggcca tcccagtgca ctgtgtgttg 120
atcaggcgcc cagtcacgcg gcctgctccc ctccacccaa ccccagggct ctatgggaag 180
gaccagcagg aggcagccct ggtggacatg gtgaatgacg gogtgaggga cctccgctgc 240
aaatacatct cctcateta caccaa 266

SEQ ID NO: 56

Length: 287

Type: DNA

Organism: Homo sapiens

16/17

Sequence: 56

tccccctgct ctcagcatat gtggggcgcc tcagtgcccc gcccaagctc aagcccttcc 60
tggcctcccc tgagtacgtg aacctcccca tcaatggcaa cgggaaacag tgagggttgg 120
ggggactctg agcggggaggc agagtttgcc ttcctttctc caggaccaat aaaatttcta 180
agagagctac tatgagcact gtgtttcctg ggaecgggct taggggttct cagcctcgag 240
gtcggtggga gggcagagca gaggactaga aaacagctcc tccagca 287

SEQ ID NO: 57

Length: 524

Type: DNA

Organism: Homo sapiens

Sequence: 57

ataaaataaa ataaaataaa ataaagcaat ttcctttcct ctaagcgccc tccacccctc 60
tccccctgcc tgtgaagcgg gtgtgcaagc tccgggatcg cagcggctctt agggaatttc 120
cccccccgat gtcccggcgc gccagttcgc tgcgcacact tcgctgcgggt cctcttccctg 180
ctgtctgttt actccctagg ccccgctggg gacctgggaa agagggaag gcttccccgg 240
ccagctgcgc ggcgactccg gggactccag ggcgcccctc tgcggccgac gcccggggtg 300
cagcggccgc cggggctggg gccggcgga gtccgggga cctccagaa gagcggccgg 360
cgccgtgact cagcactggg gcggagcggg gcgggaccac ccttataagg ctcgaggacc 420
gcgaggcctt cgctggagtt tcgccgcgc agtcctcgcc accagtgaat acgcgcggcc 480
cgctgcccg gggatggggc tcagagctcc cagcatgggg ccaa 524

SEQ ID NO: 58

Length: 524

Type: DNA

Organism: Homo sapiens

Sequence: 58

ataaaataaa ataaaataaa ataaagtaat tttttttttt ttaagtgtt tttatttttt 60
ttttttgttt tgtgaagtgg gtgtgtaagt tttgggattg tagtggtttt agggaatttt 120
ttttttgtat gttttgtgtt gttagtttgt tgtgtatatt ttgttggtgt tttttttttg 180
ttgtttgttt attttttagg ttttggtggg gatttgggaa agagggaag gtttttttgg 240
ttagttgtgt ggtgattttg gggatttttag ggtgtttttt tgtggttgat gtttgggtgt 300
tagtggttgt tggggttggg gttggtggga gtttggtggga ttttttagaa gagtgttgg 360

17/17

tgttgtgatt tagtattggg gtggagtggg gtgggattat tttataaagg tttggaggtt 420
gtgaggtttt tgttggagtt ttgttgttgt agtttttctt attagttagt atgtgtggtt 480
tgtgtttttg gggatggggt ttagagtttt tagtatgggg ttaa 524

SEQ ID NO: 59

Length: 524

Type: DNA

Organism: Homo sapiens

Sequence: 59

ataaaataaa ataaaataaa ataaagtaat tttttttttt ttaagcggtt tttatttttt 60
ttttttgttt tgtgaagcgg gtgtgtaagt ttccggatcg tagcggtttt agggaatttt 120
ttttcgcgat gtttcggcgc gttagtctgt tgcgtatat tctgtgcggt tttttttttg 180
ttgtttgttt attttttagg ttctgttggg gatttgggaa agagggaaag gttttttcgg 240
ttagttgcgc ggcgatttcg gggatttttag ggcgtttttt tgcggtcgac gttcgggggtg 300
tagcggtcgt cgggggttggg gtcggcggga gttcgcggga ttttttagaa gagcggtcgg 360
cgctgtgatt tagtattggg gcggagcggg gcgggattat tttataaagg ttccggaggtc 420
gcgaggtttt cgttggagtt tctgtctgtct agttttctgt attagttagt acgcgcgggtt 480
cgcgtttttc gggatggggt ttagagtttt tagtatgggg ttaa 524

ASSAY FOR METHYLATION IN THE GST-Pi GENEField of the Invention:

This invention relates to an assay for diagnosis or prognosis of a disease or condition characterised by abnormal methylation of cytosine at a site or sites within the glutathione-S-transferase (GST) Pi gene and/or its regulatory flanking sequences. In one particular application, the invention provides an assay for the diagnosis or prognosis of prostate cancer.

Background of the Invention:*DNA METHYLATION IN MAMMALIAN GENOMES*

The only established post-synthetic modification of DNA in higher animal and plant genomes is methylation of the 5' position of cytosine. The proportion of cytosines which are methylated can vary from a few percent in some animal genomes (1) to 30% in some plant genomes (2). Much of this methylation is found at CpG sites where the symmetrically positioned cytosines on each strand are methylated. In plant genomes, similar symmetrical methylation of cytosines at CpNpG (where N can be any base) is also common (3). Such sites of methylation have also been identified at low frequency in mammalian DNA (4).

Methylation patterns are heritable as the methylase enzyme recognises as a substrate, sites where a CpG dinucleotide is methylated on one strand but the corresponding C on the other strand is unmethylated, and proceeds to methylate it (5, 6). Fully unmethylated sites do not normally act as substrates for the enzyme and hence remain unmethylated through successive cell divisions. Thus, in the absence of errors or specific intervening events, the methylase enzyme enables the stable heritability of methylation patterns.

Extensive studies of gene expression in vertebrates have shown a strong correlation between methylation of regulatory regions of genes and

their lack of expression (7). Most of such studies have examined only a limited number of restriction enzyme sites using enzymes which fail to cut if their target sites are methylated. A far more limited number have been examined at all cytosine bases using genomic sequencing methods (8, 9).

5 *BISULPHITE CONVERSION OF DNA*

Treatment of single-stranded DNA with high concentrations of bisulphite followed by alkali leads to the selective deamination of cytosine, converting it to uracil (10, 11). By contrast, 5-methyl cytosines (5meC) are resistant to this chemical deamination. When bisulphite-treated DNA is copied by DNA polymerases, the uracils are read as if they were thymines and an adenine nucleotide incorporated, while 5meC is still read as a cytosine (a G being incorporated opposite). Thus, after a region of sequence is amplified by polymerase chain reaction (PCR), cytosines in the sequence which were methylated in the original DNA will be read as cytosines while unmethylated cytosines will be read as thymines (12, 13).

15 *PCR AMPLIFICATION OF METHYLATED AND UNMETHYLATED DNA*

In order to amplify bisulphite-treated DNA, primers are designed to anneal to the sequence produced after bisulphite treatment of the DNA. Since cytosines are converted to uracils, the base in the annealing primer will be an adenine rather than a guanine for the non-converted cytosine. Similarly, for the other primer of the pair, thymines replace cytosines. To permit quantification of levels of methylation in the target DNA, primers are normally chosen to avoid sites which may or may not be methylated (particularly CpG sites) and so may contain either a 5meC or a uracil after bisulphite treatment. Use of such non-selective primers allows both methylated and unmethylated DNAs to be amplified by PCR, providing for quantification of the level of methylation in the starting DNA population. The PCR-amplified DNA can be cut with an informative restriction enzyme, can be sequenced directly to provide an average measure of the proportion of methylation at any position or molecules may be cloned and sequenced (each

clone will be derived from amplification of an individual strand in the initial DNA). Such studies have indicated that, while a population of molecules may conform to an overall pattern of methylation, not all molecules will be identical and methylation may be found on only a fraction of molecules at some sites (13, 16).

SELECTIVE AMPLIFICATION OF METHYLATED DNA

Recently Herman *et al.* (14) described a variation of the bisulphite sequencing procedure to make it selective for the amplification of only methylated DNA. In this work, PCR primers were used which were designed to discriminate between the sequences produced after bisulphite-treatment of methylated and non-methylated target DNAs. Thus, cytosines which formed part of a CpG site would not be bisulphite converted and would remain as cytosines in the methylated DNA but would be converted to uracils in the unmethylated target DNA. Primers utilising these differences were designed and used for the amplification of methylated DNA sequences from four tumour suppressor genes, p16, p15, E-cadherin and von Hippel-Lindau.

METHYLATION OF THE GLUTATHIONE-S-TRANSFERASE π GENE IN PROSTATE CANCER

Lee *et al.* (15) (US Patent No 5,552,277 and International Patent Application No PCT/US95/09050) demonstrated that expression of the glutathione-S-transferase (GST) π gene is lost in nearly all cases of prostate cancer. They further showed that in twenty cases examined, using Southern blotting, that this loss of expression was accompanied by methylation at a specific restriction enzyme site (*Bss*HII) in the promoter region of the gene. This methylation was not seen in normal prostate tissue or in a number of other normal tissues examined. In examining a prostate cancer cell line in which the GST- π gene is inactive, they also identified methylation at two other restriction enzyme sites, *Not*I and *Sac*II in the promoter region of the gene. Digestion of cell line DNAs with the enzymes *Msp*I and *Hpa*II, indicated that the correlation of DNA methylation with lack of expression

was not maintained for these sites which were largely located downstream of the transcription start site. The nature of the data makes it difficult to reach conclusions on the methylation status of individual *MspI/HpaII* sites.

However, Lee *et al.* (18) were able to show that following *HpaII* digestion (which will cut at all unmethylated *HpaII* sites), a region of DNA containing twelve *HpaII* recognition sites could be amplified by PCR from tumour DNA, but not from normal prostate or leukocyte DNA. This indicates that some DNA molecules in prostate cancer are methylated at all these *HpaII* sites, while DNAs from normal prostate and leukocyte DNA must contain at least one of these sites unmethylated (as a single cut will render the region incapable of being amplified by PCR).

The present inventors have identified and developed an alternative method for detecting sites of methylation present in DNA from prostate cancer tissue but not present in DNA from normal tissue. The method relies on selective amplification of a target region of the GST-Pi gene but does not require prior restriction with an informative restriction enzyme.

Disclosure of the Invention:

Thus, in a first aspect, the present invention provides a diagnostic or prognostic assay for a disease or condition in a subject, said disease or condition characterised by abnormal methylation of cytosine at a site or sites within the glutathione-S-transferase (GST) Pi gene and/or its regulatory flanking sequences, wherein said assay comprises the steps of;

- (i) isolating DNA from said subject,
- (ii) exposing said isolated DNA to reactants and conditions for the amplification of a target region of the GST-Pi gene and/or its regulatory flanking sequences which includes a site or sites at which abnormal cytosine methylation characteristic of the disease or condition occurs, the amplification being selective in that it only amplifies the target region if the

said site or sites at which abnormal cytosine methylation occurs is/are methylated, and

(iii) determining the presence of amplified DNA, wherein the amplifying step (ii) is used to amplify a target region of the GST-Pi gene and/or its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +55.

Since the amplification is designed to only amplify the target region if the said site or sites at which abnormal cytosine methylation (i.e. as compared to the corresponding site or sites of DNA from subjects without the disease or condition being assayed) occurs is/are methylated, the presence of amplified DNA will be indicative of the disease or condition in the subject from which the isolated DNA has been obtained. The assay thereby provides a means for diagnosing or prognosing the disease or condition in a subject.

The step of isolating DNA may be conducted in accordance with standard protocols. The DNA may be isolated from any suitable body sample, such as cells from tissue (fresh or fixed samples), blood (including serum and plasma), semen, urine, lymph or bone marrow. For some types of body samples, particularly fluid samples such as blood, semen, urine and lymph, it may be preferred to firstly subject the sample to a process to enrich the concentration of a certain cell type (e.g. prostate cells). One suitable process for enrichment involves the separation of required cells through the use of cell-specific antibodies coupled to magnetic beads and a magnetic cell separation device.

Prior to the amplifying step, the isolated DNA is preferably treated such that unmethylated cytosines are converted to uracil or another nucleotide capable of forming a base pair with adenine while methylated cytosines are unchanged or are converted to a nucleotide capable of forming a base pair with guanine. This treatment permits the design of primers which enable the selective amplification of the target region if the said site or sites at which abnormal cytosine methylation occurs is/are methylated.

Preferably, following treatment and amplification of the isolated DNA, a test is performed to verify that unmethylated cytosines have been efficiently converted to uracil or another nucleotide capable of forming a base pair with adenine, and that methylated cytosines have remained unchanged or efficiently converted to another nucleotide capable of forming a base pair with guanine.

Preferably, the treatment of the isolated DNA involves reacting the isolated DNA with bisulphite in accordance with standard protocols. As will be clear from the above discussion of bisulphite treatment, unmethylated cytosines will be converted to uracil whereas methylated cytosines will be unchanged. Verification that unmethylated cytosines have been converted to uracil and that methylated cytosines have remained unchanged may be achieved by;

- (i) restricting an aliquot of the treated and amplified DNA with a suitable restriction enzyme(s) which recognise a restriction site(s) generated by or resistant to the bisulphite treatment, and
- (ii) assessing the restriction fragment pattern by electrophoresis.

Alternatively, verification may be achieved by differential hybridisation using specific oligonucleotides targeted to regions of the treated DNA where unmethylated cytosines would have been converted to uracil and methylated cytosines would have remained unchanged.

The amplifying step may involve polymerase chain reaction (PCR) amplification, ligase chain reaction amplification (20) and others (21).

Preferably, the amplifying step is conducted in accordance with standard protocols for PCR amplification, in which case, the reactants will typically be suitable primers, dNTPs and a thermostable DNA polymerase, and the conditions will be cycles of varying temperatures and durations to effect alternating denaturation of strand duplexes, annealing of primers (e.g. under high stringency conditions) and subsequent DNA synthesis.

To achieve selective PCR amplification with bisulphite-treated DNA, primers and conditions may be used to discriminate between a target region including a site or sites of abnormal cytosine methylation and a target region where there is no site or sites of abnormal cytosine methylation. Thus, for amplification only of a target region where the said site or sites at which abnormal cytosine methylation occurs is/are methylated, the primers used to anneal to the bisulphite-treated DNA (i.e. reverse primers) will include a guanine nucleotide(s) at a site(s) at which it will form a base pair with a methylated cytosine(s). Such primers will form a mismatch if the target region in the isolated DNA has unmethylated cytosine nucleotide(s) (which would have been converted to uracil by the bisulphite treatment) at the site or sites at which abnormal cytosine methylation occurs. The primers used for annealing to the opposite strand (i.e. the forward primers) will include a cytosine nucleotide(s) at any site(s) corresponding to site(s) of methylated cytosine in the bisulphite-treated DNA.

Preferably, the primers used for the PCR amplification are of 12 to 30 nucleotides in length and are designed to anneal to a sequence within the target region that includes two to four cytosine nucleotides that are abnormally methylated in the DNA of a subject with the disease or condition being assayed. In addition, the primers preferably include a terminal nucleotide that will form a base pair with a cytosine nucleotide (reverse primer), or the guanine nucleotide opposite (forward primer), that is abnormally methylated in the DNA of a subject with the disease or condition being assayed.

The step of amplifying is used to amplify a target region within the GST-Pi gene and/or its regulatory flanking sequences. The regulatory flanking sequences may be regarded as the flanking sequences 5' and 3' of the GST-Pi gene which include the elements that regulate, either alone or in combination with another like element, expression of the GST-Pi gene.

In particular, the step of amplifying is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +55 (wherein the numbering of the CpG sites is relative to the transcription start site). The numbering and position of CpG sites is shown in Figure 1.

The step of determining the presence of amplified DNA may be conducted in accordance with standard protocols. One convenient method involves visualisation of a band(s) corresponding to amplified DNA, following gel electrophoresis.

Preferably, the disease or condition to be assayed is selected from cancers, especially hormone dependent cancers such as prostate cancer, breast cancer and cervical cancer, and liver cancer.

For the diagnosis or prognosis of prostate cancer, the step of amplifying preferably amplifies a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +53, more preferably, -43 to +10. However, within these target regions it is believed that there are CpG sites which show variability in methylation status in prostate cancer or are methylated in other tissues. Thus, for the target region defined by (and inclusive of) CpG sites -43 to +10, it is preferred that the primers used for amplification be designed so as to minimise (i.e. by use of redundant primers or by avoidance of the sites) the influence of CpG sites -36, -32, -23, -20, -19, -14 and a polymorphic region covering site -33. Further, for DNA isolated from cells other than from prostate tissue (e.g. blood), it is preferred that the primers used be designated to amplify a target region that does not include the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -7 to +7, or, more preferably, -13 to +8, since this may lead to false positives. Further preferred target regions, therefore, are within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to -14, -43 to -8, +9 to +53 and +1 to +53.

Suitable primer pairs for the diagnosis or prognosis of prostate cancer, include those consisting of a forward and reverse primer selected from each of the following groups:

Forward Primers (i.e. anneal to the 5' end of the target region)

- 5 CGCGAGGTTTTCGTTGGAGTTTCGTCGTC (SEQ ID NO: 1)
CGTTATTAGTGAGTACGCCGCGTTC (SEQ ID NO: 2)
YGGTTTATAGGGAATTTTTTTCGC (SEQ ID NO: 3)
YGGYGYGTTAGITYGTTGYGTATATTC (SEQ ID NO: 4)
GGGAATTTTTTTTCGGATGTTTYGGCGC (SEQ ID NO: 5)
10 TTTTATAGGGGTTTGGAGCGTTTC (SEQ ID NO: 6)
GGTAGGTTGYGTTTATCGC (SEQ ID NO: 7)

Reverse Primers (i.e. anneal to the extension of the forward primer)

- TCCGATCCCTCCCCGAAACGCTCCG (SEQ ID NO: 8)
GAAACGCTCCGAACCCCTAAAAACCGCTAACG (SEQ ID NO: 9)
15 CRCCCTAAAAATCCCRAAATCRCCGCG (SEQ ID NO: 10)
ACCCRCRACRCCRTACACCCRAACGTCG (SEQ ID NO: 11)
CTCTTCTAAAAAATCCCRCAACTCCCGCG (SEQ ID NO: 12)
AAAACRCCCTAAAAATCCCGGAAATCGCCG (SEQ ID NO: 13)
AACTCCCRCCGACCCCAACCCGACGACCG (SEQ ID NO: 14)
20 AAAAATTCTAATCTCTCCGAATAAACG (SEQ ID NO: 15)
AAAAACCRAAATAAAAAACACACGACG (SEQ ID NO: 16)

wherein Y is C, T or, preferably, a mixture thereof, and R is A, G or, preferably, a mixture thereof.

- 25 For the diagnosis or prognosis of liver cancer, the step of amplifying preferably amplifies a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to -14 and/or +9 to +53. However, within these target regions it is believed that there are CpG sites which show variability in methylation status in liver cancer or are methylated in other tissues. Thus, for the target region defined
30 by (and inclusive of) CpG sites -43 to -14, it is preferred that the primers used

for amplification be designed so as to minimise (i.e. by use of redundant primers or by avoidance of the sites) the influence of CpG sites -36, -32, -23, -20, -19, -14 and a polymorphic region covering site -33.

It will be appreciated by persons skilled in the art, that a site or sites of abnormal cytosine methylation within the above identified target regions of the GST-Pi gene and/or its regulatory flanking sequences, could be detected for the purposes of diagnosing or prognosing a disease or condition (particularly, prostate cancer and/or liver cancer) by methods which do not involve selective amplification. For instance, oligonucleotide/polynucleotide probes could be designed for use in hybridisation studies (e.g. Southern blotting) with bisulphite-treated DNA which, under appropriate conditions of stringency, selectively hybridise only to DNA which includes a site or sites of abnormal methylation of cytosine(s). Alternatively, an appropriately selected informative restriction enzyme(s) could be used to produce restriction fragment patterns that distinguish between DNA which does and does not include a site or sites of abnormal methylation of cytosine(s).

Thus, in a second aspect, the present invention provides a diagnostic or prognostic assay for a disease or condition in a subject said disease or condition characterised by abnormal methylation of cytosine at a site or sites within the glutathione-S-transferase (GST) Pi gene and/or its regulatory flanking sequences, wherein said assay comprises the steps of;

- (i) isolating DNA from said subject, and
- (ii) determining the presence of abnormal methylation of cytosine at a site or sites within the region of the GST-Pi gene and/or its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +55.

The step of isolating DNA may be conducted as described above in relation to the assay of the first aspect.

Preferably, the region of the GST-Pi gene and its regulatory flanking sequences within which the presence of methylated cytosine(s) at a site or sites is determined is selected from the regions defined by (and inclusive of)

CpG sites -43 to +53, -43 to +10, -43 to -14, +9 to +53 and +1 to +53. However, within these regions, it is preferred that certain sites (namely, CpG sites, -36, -33, -32, -23, -20, -19, and -14) be avoided as the site or sites at which, for the purpose of the assay, the presence of abnormal methylation of cytosine is determined.

Where the determination step is to involve selective hybridisation of oligonucleotide/polynucleotide/peptide-nucleic acid (PNA) probes, prior to the determination step, the isolated DNA is preferably treated (e.g. with bisulphite) such that unmethylated cytosines are converted to uracil or another nucleotide capable of forming a base pair with adenine while methylated cytosines are unchanged or are converted to a nucleotide capable of forming a base pair with guanine. This treatment permits the design of probes which allow for selective hybridisation to a target region including a site or sites of abnormal methylation of cytosine.

In a third aspect, the present invention provides a primer or probe (sequence shown in the 5' to 3' direction) comprising a nucleotide sequence selected from the group consisting of:

- | | | |
|----|----------------------------------|-----------------|
| | CGCGAGGTTTTCGTTGGAGTTTCGTCGTC | (SEQ ID NO: 1) |
| | CGTTATTAGTGAGTACGCGCGGTTTC | (SEQ ID NO: 2) |
| 20 | YGGTTTTAGGGAATTTTTTTTCGC | (SEQ ID NO: 3) |
| | YGGYGYGTIAGTTYGTTGYGTATATTTTC | (SEQ ID NO: 4) |
| | GGGAATTTTTTTTCGCGATGTTTYGGCGC | (SEQ ID NO: 5) |
| | TTTTTAGGGGGTTYGGAGCGTTTC | (SEQ ID NO: 6) |
| | GGTAGGTTGYGTTTATCGC | (SEQ ID NO: 7) |
| 25 | AAAAATTCTAATCTCTCCGAATAAACG | (SEQ ID NO: 8) |
| | AAAAACCRAAATAAAAACCACACGACG | (SEQ ID NO: 9) |
| | TCCCATCCCTCCCGAAACGCTCCG | (SEQ ID NO: 10) |
| | GAAACGCTCCGAACCCCTAAAAACCGCTAACG | (SEQ ID NO: 11) |
| | CRCCCTAAAAATCCCCRAAATCRCCGCG | (SEQ ID NO: 12) |
| 30 | ACCCCRACRACCRCTACACCCRAACGTCG | (SEQ ID NO: 13) |

CTCTTCTAAAAAATCCCRCAACTCCGCGG (SEQ ID NO: 14)

AAAACRCCTAAAAATCCCGGAAATCGCGG (SEQ ID NO: 15)

AACTCCRCGCGACCCCAACCCGACGACCG, (SEQ ID NO: 16)

wherein Y is C, T or, preferably, a mixture thereof, and R is A, G or,

5 preferably, a mixture thereof.

The terms "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated component, feature or step or group of components, features or steps with or without the inclusion of a further component, feature or step or group of components. features or steps.

The invention will now be further described with reference to the accompanying figures and following, non-limiting examples.

Brief description of the accompanying figures:

15 *Figure 1* shows the organisation and nucleotide sequence of the human GST-Pi gene. CpG sites are numbered relative to the transcription start site. Nucleotide Sequence numbering is according to the GST-Pi gene sequence of Genbank Accession No. M24485.

20 *Figure 2* shows the region of the GST-Pi gene exhibiting differential methylation in prostate cancer. The figure further shows the sequence and derivation of primers for the upstream region (from CpG site -43 to +10) and the common polymorphism encompassing CpG site -33 (shown above the sequence (p)). Underneath the GST-Pi sequence is shown the sequence of the derived strand after conversion of cytosines to uracil. The derived strand
25 is shown either assuming all CpGs are methylated (B-M) or un-methylated (B-U). Below this is shown specific primers designed to selectively amplify the methylated sequence.

Figure 3 shows the methylation status of each CpG site in isolated DNAs;

A - for the core promoter region through to the 3' end of the GST-Pi gene for the LNCaP (LN) cell line, DU145 (DU) cell line, PC3 cell line, PC3-M cell line and PC3-MM cell line, for DNA isolated from normal tissue samples from prostate cancer patients (2AN, BN and CN), for prostate tumour tissue (BC, CC, DC, XC, WC and 2AC) and for normal prostate tissue (Pr) from a person without prostate cancer:

B - for the core promoter region and upstream sequences of the GST-Pi gene from normal prostate tissue (from a person without prostate cancer), from three prostate cancer samples (BC, CC and DC) and for a number of other normal tissues. Patients B and D were polymorphic at CpG site -33 and the level of methylation indicated in the brackets reflects methylation of the allele which contains the CpG. For CpG sites -28 to +10, the level of methylation was determined by direct sequence analysis of the population of PCR molecules (17). For the upstream CpG sites, -56 to -30, PCR products were cloned and a number of individual clones sequenced (number indicated in brackets below the sample name). For normal tissues the level of methylation at each site was determined as the fraction of all clones containing a C at that position. For the cancer samples BC, CC and DC, the level of methylation shown is that among the clones which showed DNA methylation in the region from CpG site -43 to -30 (about half of the clones in each case).

In both A and B, a blank box indicates that the site was not assayed, and a "B" indicates that the status of the site could not be determined (e.g. because of a sequence blockage or it was beyond the range of the sequencing run). The level of methylation detected at each site is shown, none (-), up to 25% (+), 26-50% (++), 51-75% (+++) and 76-100% (++++) . The Gleason Grade of tumour samples is also shown.

Figure 4 provides the results of amplification of bisulphite treated DNAs from a variety of tissues;

A - panel A (region covering the transcription start site) used CGPS-1 and 3 as outer primers and CGPS-2 and 4 as inner primers, Panel B used the outer primer pair CGPS-5 and 8 which encompass the region from CpG site -39 to -16 for first round amplification, followed by a second round of amplification with the CGPS-6 and 7 primers, amplifying a 140 bp fragment covering CpG sites -36 to -23. The lanes are 1. Brain, 2. Lung, 3. Skeletal muscle, 4. Spleen, 5. Pancreas, 6. "Normal" Prostate Aged 85 y.o., 7. "Normal" Prostate Aged 62 y.o., 8. Heart, 9. Bone Marrow, 10. Blood-1, 11. Blood-2, 12. Blood-3, 13. Liver-1, 14. Liver-2;

B - used the same primer pairs as that of the amplification shown in Figure 4A Panel B. with DNA from 10 prostate cancer tissue samples (c) and matched normal (n) tissue samples from the same prostates (a positive control (+) LNCaP DNA and a negative control (-) is also shown). Underneath is the Gleason grade and the level of methylation of samples seen with non-selective primers.

C - used the same primer pairs as that of the amplification shown in Figure 4A Panel B, with DNA from a range of healthy tissues, blood from prostate cancer patients and various cell lines. The lanes are: Panel A 1-10 blood samples from prostate cancer patients during radical prostatectomy; Panel B 1. normal prostate-1, 2. normal prostate-2, 3. normal prostate-3, 4. normal prostate-4, 5. normal prostate-5, 6. HPV transformed prostate cell line, 7. blood from prostate patient PA (PSA=1000), 8. blood from prostate patient PB (PSA=56), 9. blood from prostate patient PC (PSA=18); and Panel C 1. LNCaP cell line, 2. Du145 cell line, 3. PC-3 cell line, 4. PC-3M cell line, 5. PC-3MM cell line, 6. Hela cell line, 7. leukemic DNA, 8. HepG2 cell line, 9. human liver DNA, 10. white blood cells, 11. MRC-5 cell line.

Figure 5 provides the results of amplification of bisulphite treated DNAs from seminal fluid of prostate cancer patients (c) and from men with

no diagnosed prostate cancer (n), using the outer primer pair CGPS-5 and 8 and CGPS-6 and 7 as the inner primer pair. The lanes are L. LNCaP cell line (positive control), D. DU145 cell line, P. PC-3 cell line (negative controls), and M. molecular weight markers.

5 *Figure 6* shows the results of amplification of bisulphite treated DNAs, wherein the DNA has been isolated from prostate tissue slides that had been identified as either cancerous or diseased with benign hyperplasia (BPH). Selective PCR amplification was conducted using the outer primer pair CGPS-5 and 8 and the inner primer pair CGPS-11 and 12.

10 *Figure 7* shows the results of amplification of bisulphite treated DNAs, wherein the DNA has been isolated from prostate cancer cells enriched from blood samples using magnetic beads coated with an anti-epithelial antibody. Different numbers of LNCaP prostate cancer cells were added to the blood samples (7A) or blood with added LNCaP cells stored for different times at 4°C or room temperature prior to DNA isolation.

15 *Figure 8* provides the results of amplification of bisulphite treated DNAs. wherein the DNA has been isolated from blood samples from normal subjects with no known prostate complaint, from patients with benign hyperplasia (BPH) of the prostate and from patients with histologically confirmed prostate cancer.

20 *Figure 9* shows the results of amplification of bisulphite treated DNAs isolated from 20 liver cancer tissue samples. Selective PCR amplification was conducted using the outer primer pair CGPS-5 and 8 and the inner primer pair CGPS-11 and 12.

25 *Figure 10* shows the results of tests conducted to confirm that any amplified DNA products has occurred from amplification of bisulphite treated DNA wherein all unmethylated cytosine has been converted to uracil. The tests are conducted using oligonucleotides probes designed to hybridise to converted or non-converted target regions.

*GENERAL METHODS AND STRATEGIES**(1) Treatment of DNA with bisulphite*

DNA for assaying was isolated from suitable sources by standard protocols and treated with bisulphite by well known methods (12, 13, 16).

5 *(2) Characterisation of Methylation of Individual Sites in DNA*

In order to determine the methylation status of individual cytosine nucleotides in target and non-target DNAs and to identify differences between them, bisulphite-modified DNA was amplified by PCR using primers designed to minimise the possibility that the methylation status of a particular CpG site will influence primer annealing and subsequent amplification (12, 13, 16).

10 *(3) Design of Selective Primers*

Based on the sequencing information, primers for use in the assay were designed to maximise the possibility that the methylation status of a particular CpG site would influence primer annealing and subsequent amplification. Specifically, the design principles followed (described for the "forward" PCR primer where the primer contains the same C to T (or U) conversions as would occur in the bisulphite-treated DNA), are listed below at (a) to (d):

- 20 (a) That primers should cover sequence regions which contain a number of C's. Conversion of unmethylated C's to U's provides for discrimination between molecules which have undergone efficient bisulphite conversion and molecules in which C's have not reacted (e.g. because not completely dissolved or containing regions of secondary structure).
- 25 (b) That at least one, but preferably at least two to four, of the C's in the regions should be C's (generally at CpG sites) known to be methylated in a high proportion of the DNA to be detected (i.e. target DNA). Thus, these C's will remain C's in the target DNA while being converted to U's in the non-target DNA. A primer which is designed to be the exactly equivalent of the
- 30 bisulphite-converted methylated DNA will contain a mismatch at each of the

positions of an unmethylated C which has been converted to a U in an unmethylated DNA. The more mismatches that are present, the greater the differential hybridisation stability of the primers will be and hence the greater the selective difference in PCR.

5 (c) That the 3' terminal base of the primer should preferably be a C corresponding to a C known to be methylated in the target DNA (normally part of a CpG dinucleotide). Correct pairing with the terminal base of the primer will provide for highly selective priming of target sequences compared with unmethylated background sequences which will form a C:A mismatch.

10 (d) That at positions where it is known that methylation occurs in only a fraction of molecules in the methylated target DNA or where it is known to vary between target DNAs (e.g. in different tumour samples), redundancy can be incorporated into the primers to allow for amplification of either C or T from the target DNA. This same approach can be used if polymorphisms are known to exist in the primer region.

15 For the "reverse" primer, which anneals to the converted strand, A's replace G's at positions opposite converted C's

(4) Verification of Selective Target Sequence Amplification

20 The amplified PCR band can be analysed to verify that it has been derived from DNA which has been fully bisulphite-converted (i.e. C's not methylated in the original DNA have been converted to U's and amplified as T's) and to further verify that the amplified DNA has been derived from the specific target DNA sequence and has the expected methylation profile (i.e. 5mC's not converted to T's). Methods for conducting these verifications include:

25 (a) Using restriction enzyme digestion.

In order to verify complete conversion, particular restriction enzymes can be used to cut the DNA. The sequence recognition sites should have the property that they contain no C's and are present in the sequence of the amplified strand after but not before bisulphite treatment. Thus, the

30

conversion of one or preferably two or more C's to U's and their amplification as T's in the PCR product should produce a new restriction site. Useful enzymes are shown in *italics* in Table 1 below.

In order to verify that the target DNA sequence amplified was specifically methylated, use can be made of restriction enzyme sites whose only C nucleotides are found as CpG dinucleotides and which, if the sequence was methylated, would remain as CpG's in the PCR products. Examples of such enzymes are shown in **bold** in Table 1 below. *BsmBI*, which cuts the non-symmetrical sequence GAGACG can also be used.

In some instances, enzymes which contain a C as an outer base in their recognition sequence can be used for verification of methylation: e.g. *EcoRI* (GAATTC) for a GAATTCG sequence or *Sau3AI* (GATC) for a GATCG sequence (**bold and underlined** in Table 1). If a site such as one of the above is present in the predicted methylated, fully bisulphite-converted DNA then the enzyme will cut the DNA only if the original CpG dinucleotide was methylated, confirming the amplification of a methylated region of DNA. Some of the enzymes (**bold and underlined** in Table 1) have the potential to be used both for monitoring efficient conversion and CpG methylation.

(b) Differential hybridisation to specific oligonucleotides.

Differential hybridisation to specific oligonucleotides can be used to discriminate that the amplified DNA is fully reacted with bisulphite and of the expected methylation profile. To demonstrate complete conversion, a pair of oligonucleotides corresponding to the same region within the amplified sequence is prepared. One oligonucleotide contains T's at all C's which should be converted by bisulphite, while the other contains C's in these positions. The oligonucleotides should contain at least two or three of such discriminatory C's and conditions be determined which provide for selective hybridisation of each to its target sequence. Similar oligonucleotides with C or T at CpG sites and T's replacing all non-CpG C's are used to determine whether the specific CpG sites are methylated.

Additional control oligonucleotides that contain no discriminatory C's, that is, either no C's or a minimal number where C's are substituted with Y's (mixture of C and T), are used to monitor the amount of PCR product in the sample. The oligonucleotides can be used for direct hybridisation detection of amplified sequences or used to select out target molecules from the PCR-amplified DNA population for other detection methods. An array of such oligonucleotides on a DNA sequencing chip can be used to establish the sequence of the amplified DNA throughout the sequence region.

(c) Single nucleotide primer extension (SNUPE).

The technique of single nucleotide primer extension can be applied to the PCR products to determine whether specific sites within the amplified sequence contain C or T bases. In this method, a primer abutting the position of interest is annealed to the PCR product and primer extension reactions performed using either just dCTP or just dTTP. The products can be separated by gel electrophoresis and quantitated to determine the proportion of each nucleotide in the population at that position. Primers should be designed to quantitate conversion of C's in CpG sites and control C's which should not be methylated. More than one primer can be included in a single reaction and/or run in the same gel track as long as their sizes can be clearly distinguished.

(d) Fluorescent Real-time Monitoring of PCR.

Oligonucleotides internal to the amplified region can be used to monitor and quantify the amplification reaction at the same time as demonstrating amplification of the correct sequence. In the Fluorogenic 5' Nuclease PCR assay (19) the amplification reaction is monitored using a primer which binds internally within the amplified sequence and which contains both a fluorogenic reporter and a quencher. When this probe is bound to its target DNA it can be cleaved by the 5' nuclease activity of the Taq polymerase, separating the reporter and the quencher. By utilising in the assay an oligonucleotide which is selective for the fully bisulphite-converted

sequence (and/or its methylation state) both the level of amplification and its specificity can be monitored in a single reaction. Other related systems that similarly detect PCR products by hybridisation can also be used.

Example 1: Methylation sequence profile of target and non-target GST-Pi DNA

MATERIALS AND METHODS

Figure 1 shows the organisation of the GST-Pi gene and the regions for which genomic sequencing was used to determine the methylation status of DNA isolated from prostate cancer tissue or cell lines and from normal prostate or other tissues. The nucleotide sequence numbering in Figure 1 is according to the GST-Pi sequence, Genbank Accession No. M24485. Also shown, within the boxes is the sequence of each amplified region, with all the CpG sites indicated and numbered relative to the position of the transcription start site. Sequence analysis demonstrated that there was an additional CpG dinucleotide (+9) not predicted from the published sequence. Also identified in the regions sequenced was a polymorphism which is present in a significant fraction of the samples studied. The polymorphic allele does not contain CpG site -33. Both the additional CpG dinucleotide and the polymorphism are shown in Figure 2. The nucleotide coordinates in Figure 2 are shown relative to the transcription start site; the first base shown, -434, corresponds to base 781 of the Genbank sequence, while the last +90, corresponds to base 1313 of the Genbank sequence.

Table 2 lists the sequences and positions of the non-selective primers used for amplification (Table 2-1) and direct sequencing (Table 2-2) of bisulphite-treated DNA.

DNA isolated from normal prostate tissue, prostate cancer tissue, prostate cancer-derived cell lines and other tissues was bisulphite treated and PCR reactions done by standard procedures (13). PCR products were either

digested with informative restriction enzymes, sequenced directly (17), or individual molecules cloned and sequenced by standard procedures.

RESULTS

In Figure 3A, the methylation status of sites in DNA from prostate cancer cell lines, prostate cancer tissue samples and matched normal prostate tissue are shown for the core promoter regions through to the 3' end of the gene (covering CpG sites -28 to 103). It can be seen that in normal prostate tissue, the core promoter region is unmethylated at all sites and that this lack of methylation extends through the region flanking the promoter to CpG site +33. Results of restriction enzyme digests of bisulphite-treated, PCR-amplified DNA indicate that this lack of methylation includes CpG sites +52 and +53. However, in the regions further downstream which were analysed, CpG sites +68 to +74 and +96 to +103, DNA from normal prostate tissue was heavily methylated. Analysis of the prostate cancer cell line LNCaP and prostate cancer tissue samples demonstrates extensive methylation of the core promoter region; variations in the overall level of methylation probably reflect the presence of different levels of normal cells within the tumour samples. DNA from one cancer sample (2AC) was found to be completely unmethylated and in contrast to the other tumour samples this tumour was found by immunohistochemistry to still be expressing GST-Pi. Sequencing of the region flanking the core promoter in the LNCaP cell line and tumour DNAs, BC and CC, showed that methylation extended through to CpG site +33 and further restriction enzyme analysis showed that methylation included CpG sites +52 and +53. For one tumour sample, DC, methylation did not extend beyond the core promoter region and CpG sites +13 to +33, as well as CpG sites +52 and +53 were found to be unmethylated. It is notable that this tumour was of Gleason Grade 2+2, the lowest grade tumour among those analysed. For all tumour DNA samples, as for the normal DNA, the downstream regions of the gene, sites 68 to 74 and 96 to 103, were heavily methylated. Within the promoter regions which were methylated in

the cancer, but not normal, tissue specific individual sites were evident which were either unmethylated or methylated to a much lower degree than surrounding methylated sites. These include sites -22 and -23 (XC), -20 (PC3 lines, XC and WC), -14 (PC3, XC and WC), +24 (PC3-M and MM2, CC), +25 (LNCaP, PC3-MM2, CC).

The results shown in Figure 3B provides a comparison of the methylation state of the core promoter region and sequences upstream of the core promoter region in DNA isolated from normal prostate tissue and from a number of other normal tissues. Sequences from the PCR fragment upstream of the core promoter were determined by cloning and sequencing as the region is refractory to direct sequencing. For the cancer samples, the level of methylation shown is as a proportion of those clones which were methylated (about 50% of the total clones in both cases). In normal prostate tissue as well as in all other normal tissues there is extensive methylation of CpG sites upstream of the AT-rich repeat. Downstream of the repeat (from CpG site -43) minimal methylation was seen in all normal tissues except normal liver tissue, where there was significant methylation of CpG sites -7 through to +7. Sequences upstream of the core promoter were found to be heavily methylated in the prostate cancer DNAs, though again specific sites were undermethylated; site -32 in cancers B and D and site -36 in cancer B.

The results therefore allow for the identification of a region of the GST-Pi gene and its regulatory flanking sequences, stretching from 3' of the polymorphic repeat region, (CpG site -43) to sites +52 and +53, which is not methylated in normal prostate tissue but is normally highly methylated in prostate cancer. In one cancer sample (D, the cancer of lowest Gleason Grade) the region from CpG sites +13 to +53 was not methylated. The more restricted region extending from CpG site -43 to +10 was methylated in all of the prostate cancer DNAs which showed promoter methylation. Methylation of part of the promoter region (CpG sites -7 to +7) was also seen in one normal tissue (liver) examined. Analysis of further samples of normal liver

DNA has shown that the level of methylation is variable and can include CpG sites from -13 to +8.

DISCUSSION

The above results are critical in identifying regions within the GST-Pi gene and/or its regulatory flanking sequences which can be used for the development of assays for the selective detection of prostate cancer cells. Thus, the region from CpG sites -43 to +53 lying within the boundary of regions methylated in normal prostate tissue can be used for the design of primers to detect cancer-specific methylation in prostate tissue samples. The region from CpG site -43 to +10 is preferred for the detection of a higher proportion of cancers. The region from CpG sites +13 to +53 may be used to detect cancer but also may be used to distinguish early (unmethylated) cancer from later (methylated cancer). For assays using other samples, such as blood, it is preferred to restrict the region chosen to exclude CpG sites -7 to +7 or, more preferably sites -13 to +8. For example, liver cells may be present in the blood taken from a subject suffering liver disease, in which case, a false positive result could be obtained if the region chosen for detection of cancer-specific methylation includes CpG sites -13 to +8.

Example 2: Design and use of selective primers for detection of methylated GST-Pi DNA

MATERIALS AND METHODS

Sequence primers for the detection of methylated GST-Pi sequences from three regions, namely a region upstream of the core promoter (primers CGPS-5 to 9 and CGPS-11 to 13), a region partially encompassing the core promoter (primers CGPS-1 to 4), and a region further downstream from the core promoter (primers CGPS-21 to 24) are shown in Table 3 below.

The sequence and derivation of primers for the upstream region are shown in Figure 2 (from CpG site -43 to CpG site +10), which also shows the common polymorphism encompassing CpG site -33 (see above the sequence

(p)). Underneath is shown the sequence of the derived strand after conversion of cytosines to uracil. The derived strand is shown either assuming all CpGs are methylated (B-M) or that none are (B-U). Below this is shown specific primers designed to selectively amplify the methylated sequence. It can be seen that all primers are designed to match perfectly to the treated, methylated template, but contain mismatches to the template derived from unmethylated DNA or the original untreated DNA. Primers CGPS-5, 8, 11, 12 and 13 are designed to avoid the polymorphic region and CpG sites which show a lower frequency of methylation in prostate cancer DNAs. The underlined T's in the forward primers (and A's in the reverse primers) derive from bisulphite conversion of C's and provide discrimination against amplification of DNA which has not been efficiently converted by the bisulphite treatment. The bold C's in the forward primers (and G's in the reverse primers) are parts of CpG sites and will form base pairs with DNA derived from methylated sequences but form mismatches to DNA derived from unmethylated sequences. Redundancy is included in some positions, Y (= mix of C and T) in forward primers and R (= mix of A and G) in reverse primers to allow pairing independent of methylation status. This can allow for certain sites where the frequency of methylation within or between tumour samples is variable (eg. site -14). Forward and reverse primers for specific selective amplification of methylated GST-Pi sequences are shown in Table 3 below.

Amplifications conducted for this example, utilised bisulphite treated DNAs from a variety of tissues and used two sets of PCR primers. Specifically, for the amplification reactions shown in Figure 4A Panel A (region covering the transcription start site), CGPS-1 and 3 were used as outer primers and CGPS-2 and 4 as inner primers. For the amplification reactions shown in Figure 4A Panel B and Figure 4B and 4C, the outer primer pair, CGPS-5 and CGPS-8 which encompass the region from CpG site

-39 to -16, were used for first round amplification, followed by second round amplification with the CGPS-6 and CGPS-7 primers, resulting in the amplification of a 140 bp fragment covering CpG sites -36 to -23. For the amplification reactions shown in Figures 5 to 8, the primer set used for the upstream region was the outer primer pair, CGPS-5 and CGPS-8, for first round amplification and the inner primer pair, CGPS-11 and CGPS-12, for second round amplification, resulting in the amplification of a 167 bp fragment covering CpG sites -38 to -23.

For all sets of primers, PCR amplifications were performed in a buffer consisting of 67 mM Tris/HCl, 16.6 mM ammonium sulphate, 1.7 mg/ml BSA and 1.5 mM MgCl₂, prepared in TE buffer (10 mM Tris/HCl pH 8.8, 0.1 mM EDTA). Reaction mixes (50 µl) contained 200 µM of each of the four dNTPs, 6 ng/ml of each primer and 2 units of AmpliTaq DNA polymerase (Perkin Elmer). For the primers CGPS-5 and 8 (first round amplification), PCR cycle conditions were 5 cycles of 60°C 1 min., 72°C 2 min. and 95°C 1 min., followed by 30 cycles of 65°C 1 min., 72°C 1.5 min. and 95°C 1 min. Amplification conditions for the primers CGPS-6 and 7 (second round amplification) were 5 cycles of 65°C 1 min., 72°C 2 min. and 95°C 1 min., followed by 30 cycles of 65°C 1 min., 72°C 1.5 min. and 95°C 1 min. For the primers CGPS-11 and 12, the amplification conditions were the same as for the CGPS-6 and 7 primers except that the annealing temperature was raised from 65°C to 70°C. 2 µl of the first round amplification reactions were used in 50 µl of second round amplification reactions. Other buffers or PCR amplification conditions may also be used to achieve similar efficiency and specificity.

RESULTS AND DISCUSSION

For the primers covering the core promoter region (see Figure 4A Panel A), amplified DNA (see arrowed band) was obtained from the positive control DNA (cancer B) but also from DNA from prostate tissue samples from two subjects who had not been diagnosed with prostate cancer. Bands of

amplified DNA were also seen from DNA isolated from a bone marrow and blood sample as well as from DNA isolated from liver tissue samples from subjects with no known prostate cancer.

For the upstream amplification (see Figure 4A Panel B), no amplified DNA was obtained from amplification reactions conducted on DNA isolated from a range of healthy tissue samples nor from DNA isolated from blood samples of subjects with no known prostate cancer; a band of amplified DNA was produced from the positive control DNA (cancer B). However, while amplification reactions conducted on DNA isolated from one normal prostate tissue sample did not result in amplified DNA, amplified DNA did result from the same amplification reactions conducted on DNA isolated from a prostate tissue sample of an 82 year old subject with no known prostate cancer. It is possible that this subject had undiagnosed prostate cancer. DNA isolated from five other samples of normal prostate tissue from subjects with no known prostate cancer did not give rise to an amplified DNA product (see Figure 4C Panel B).

In Figure 4B, the results of PCR amplification reactions are shown for tissue samples from patients with prostate cancer: for each sample, DNA was isolated from a region identified as containing cancer and from another region identified as grossly normal. In all cases, a clear band of amplified DNA was produced from amplification reactions conducted on prostate cancer DNA. Two of these, were cases where the proportion of methylated DNA was insufficient to be detected using primers designed to prime equivalently on methylated and unmethylated DNA. For DNA isolated from grossly normal tissue, the band of amplified DNA was either absent or present in a substantially lower amount. The presence of a band in some "normal" samples could derive from a low level of cancer cells in the sample.

Amplification of DNA from samples of blood obtained from the abdominal cavity during surgery showed that it was possible to detect methylated GST-Pi sequences in a number of them. Samples of peripheral

blood isolated from three patients with known metastatic disease (see Fig 4C Panel B) demonstrated the presence of amplifiable, methylated GST-Pi sequences.

Amplified DNA products were also produced from amplification of DNA isolated from the LNCaP and DU145 prostate cancer cell lines, but not from the PC-3 series of cell lines. This latter result could be due to a low level of methylation in the upstream promoter region in PC-3 cells, but a major contributing factor is likely to be a lack of priming by the CGPS-6 primer as PC-3 only contains the variant allele of the GST-Pi gene.

Methylated GST-Pi sequences were also detected in DNA isolated from some tumour-derived cell lines of non-prostatic origin: HeLa, a cervical carcinoma, and HepG2, a liver carcinoma (see Figure 4C Panel B).

DNA was isolated from the seminal fluid (see Figure 5) of 3 prostate cancer patients (C) and from 5 subjects with no known prostate cancer (N), treated with bisulphite and amplified using primers CGPS-5 and 8 followed by CGPS-6 and 7. Amplified DNA products were obtained from all three cancer DNAs. One of the five samples from subjects without diagnosed prostate cancer also resulted in an amplified DNA product, but it is not clear if this represents a false positive or a case of undiagnosed prostate cancer in the particular subject.

The use of the primer CGPS-11 avoids annealing across the polymorphic sequence at CpG site -33, and the combination of CGPS-5 and 8 as outer primers followed by CGPS-11 and 12 as inner primers was found to give efficient amplification of prostate cancer DNA. In a first experiment (see Figure 6), DNA was extracted from regions of fixed tissue slides that had been identified as either being cancerous or being diseased with benign hyperplasia (BPH). DNA was isolated by incubating scraped material in 400 µl of 7M guanidinium hydrochloride, 5 mM EDTA, 100 mM Tris/HCl pH 6.4, 1% Triton-X100, 50 mg/ml proteinase K and 100 mg/ml yeast tRNA. After homogenisation, samples were incubated for 48 hours at 55°C then subjected

to five freeze/thaw cycles of dry ice for 5 min./95°C for 5 min. After vortexing and centrifugation for 2 min. in a microfuge, the supernatants were then diluted three fold, extracted with phenol/chloroform and ethanol precipitated. DNA isolated from samples from 6 cancer patients and 4 with BPH were amplified with either non-selective primers for the core promoter region (i.e. control PCR amplification with GST-9 and 10 followed by GST-11 and 12) or CG selective primers (i.e. selective PCR amplification with CGPS-5 and 8 followed by CGPS-11 and 12). Control PCR amplifications demonstrated the presence of amplifiable DNA in all samples. Using the CG selective primers, amplified DNA products were only obtained from the cancer DNAs. The PSA (prostate specific antigen) levels of these patients ranged from 4 to 145 ng/ml. For the BPH patients, the PSA levels ranged from 2.3 to 25 ng/ml.

In further experiments, prostate cancer cells were first enriched from blood samples using antibodies coupled to magnetic beads followed by DNA isolation, bisulphite modification and PCR amplification. Cell isolation was achieved using Dynabeads anti-Epithelial Cell (Dynal Prod. No. 112.07) essentially as described by the manufacturer. The magnetic beads were coated with the anti-epithelial antibody mAb Ber-EP4 (22). Alternatively, magnetic beads coupled to antibodies specific for the extracellular domain of the prostate specific membrane antigen (23) could have been used. Whole blood was diluted 1:1 with Dulbecco's phosphate buffered saline (PBS) containing 10 mM EDTA and 40 µl of pre-washed magnetic beads added. Cells were incubated at 4°C on a rotating platform for 30 min and then the beads were collected to the side of the tube using a magnetic cell separation device for 4 min. The supernatant was then carefully aspirated and the beads resuspended in the washing solution (PBS containing 0.5% bovine serum albumin). Beads were then again collected to the side of the tube using a magnet and the supernatant carefully aspirated before conducting a further wash was done with the tube remaining in place in the magnetic

separation device and the supernatant aspirated. The beads were then resuspended in DNA isolation buffer (100 mM Tris/HCl pH 8, 25 mM EDTA, 1% Sarkosyl. 200 mg/ml proteinase K), incubated for at least 2 h at 37° and DNA recovered by phenol/chloroform extraction and ethanol precipitation. The DNA was then finally subjected to bisulphite treatment and PCR amplification.

The sensitivity of this method was tested by seeding varying numbers of cells of a prostate cancer cell line, LNCaP, into normal blood. As shown in Figure 7A, the presence of 20 cells or more in 0.5 ml of blood could be reliably detected. The experiment shown in Figure 7B showed that blood samples containing LNCaP cells could be stored at room temperature or at 4 °C for up to 24 hours without loss of sensitivity.

Using magnetic bead capture followed by bisulphite treatment and selective PCR amplification, patient blood samples were also analysed and the results from a set of these are shown in Figure 8. These include blood samples from normal subjects with no known prostate complaint, from patients with benign hyperplasia (BPH) of the prostate and from patients with histologically confirmed prostate cancer. The control PCR amplifications (upper panel) used primers which amplify both methylated and unmethylated GST-Pi sequences. The amplifications using CG-selective primers are shown in the lower panel. Positive control amplifications (LNCaP (L) and PC3 (P)) are shown in the cancer panels and negative control amplifications are shown in the normal and cancer panels.

Table 4 below summarises the results of testing of DNA from patient blood samples using the magnetic bead/CG selective PCR amplification protocol. No amplified DNA products were obtained from DNA isolated from normal control subjects, and only DNA isolated from one of 18 patients diagnosed histologically to have BPH produced amplified DNA products (this patient had a blood PSA level of 17 ng/ml). Of patients with confirmed prostate cancer, isolated DNA from 17 of 24 (70%) were PCR-positive (i.e.

resulted in the production of amplified DNA), indicating the presence of prostate cancer cells in the blood. For patients clinically staged as A and B, (i.e. disease confined to the prostate), cancer cells were detected in the blood in 6 of the 10 cases. For 9 patients with locally invasive (Stage C) or metastatic (Stage D) disease, cancer cells were detected in the blood in every case.

Since it was found that the HepG2 liver cancer cell line contained methylated GST-Pi sequences, samples of DNA isolated from liver cancer tissue was also examined. DNA isolated from 20 liver cancer samples were bisulphite treated and amplified using the CGPS-5 and 8 and CGPS-11 and 12 primer pairs (see Figure 9). 14 of the 20 samples were PCR-positive. On the other hand, no amplified DNA products were produced from DNA isolated from 2 patients with no liver cancer (see Figure 4 and data not shown). DNA isolated from normal liver tissue was shown to be partially methylated in the region of the transcription start site (CpG sites -7 to +7, see Figure 3B). Analysis of further samples of normal liver DNA has shown that the level of methylation is variable and can include CpG sites from -13 to +8. The primer pairs used here encompass CpG sites -39 to -16, upstream of the region of methylation seen in normal liver DNA.

The above results show that different sets of primers designed to hybridise the core promoter of the GST-Pi gene or the region upstream of the core promoter, can reliably amplify bisulphite-treated DNA that has been isolated from prostate cancer cells. However, primers designed to hybridise to the core promoter are less selective in that DNAs isolated from a number of normal tissue samples result in amplified DNA products. Thus, primers designed to hybridise to regions found to be unmethylated in DNA from normal tissues, that is, the upstream region encompassing CpG sites -45 to -8 and the region downstream of the promoter encompassing CpG sites +8 to +53, are preferred for the prognostic or diagnostic assaying of prostate

cancer. Additionally, primers designed to hybridise to this latter region may also be useful for discriminating between early and late prostate cancer.

Example 3: Confirmation of correct amplification

The specific oligonucleotides probes described below can be used to confirm that any amplified DNA products resulting from the amplification step of the assay is due to DNA in which all unmethylated cytosines had been converted to uracils. Those for the upstream PCR region can be used with amplified DNA products from all combinations of the CGPS-5, 6, 11, 7 to 9, 12 and 13 forward and reverse primers. Those for the downstream PCR region can be used with amplified DNA products of the CGPS-21 to 24 primers. A biotinylated version of the conversion-specific oligonucleotide can also be used for the selective and specific capture from solution of the amplified DNA products generated using these primer pairs, or the appropriately labelled oligonucleotide can be used for real-time monitoring of specific PCR fragment amplification. Amplified DNA products from PCR amplification of bisulphite-treated DNA routinely have one strand containing a very high proportion of thymine nucleotides and the other strand containing a very high proportion of adenine nucleotides. Because of this, it is possible to use oligo dT (or oligo dA) as a generic conversion specific oligonucleotide, the annealing conditions being varied to optimise discrimination of converted and non-converted DNA for each PCR fragment.

Upstream PCR region:

Conversion oligonucleotide:

HybC5 5'-AAACCTAAAAAATAAACAAACAA (SEQ ID NO: 17)

Non-conversion oligonucleotide:

HybU5 5'-GGGCCTAGGGAGTAAACAGACAG (SEQ ID NO: 18)

Conversion neutral oligonucleotide:

HybN5: 5'-CCTTTCCCTCTTCCCAARTCCCCA (SEQ ID NO: 19)

Downstream PCR region:

Conversion oligonucleotide:

HybC3 5'-TTTGGTATTTTTTCGGGTTTAG (SEQ ID NO: 20)

5 Non-conversion oligonucleotide:

HybU3 5'-CTTGGCATCCTCCCCGGGCTCCAG (SEQ ID NO: 21)

Conversion neutral oligonucleotide:

HybN3 5'-GGYAGGGAAGGGAGGYAGGGYTGGG (SEQ ID NO: 22)

10 To demonstrate the selectivity of such hybridisations, a series of DNAs were spotted onto nylon membranes and hybridised with conversion and non-conversion specific oligonucleotide probes for the upstream PCR region as well as a control oligonucleotide. The DNAs included:

15 (i) individual cloned PCR products from amplification of the upstream region that contained differing numbers of converted cytosines in the region complementary to the probe (see Figure 10, where the number of converted cytosines, out of 10, is shown (Column 1 and top 2 spots of Column 2). n.b. the two clones containing 10/10 converted bases end adjacent to and do not contain the sequences complementary to the control oligonucleotide); and

20 (ii) PCR products from cancer patients and patients with benign hyperplasia that had been amplified from bisulphite-treated DNA using CG-selective primers (CGPS-5 and 8, followed by CGPS 11 and 12) (see figure 10, where these are labelled as Cancer Samples 1 to 4 (lower part of column 2) and BPH samples 1 to 4 (Column 3)).

25 Hybridisations with kinased oligonucleotide probes were performed in Express-Hyb buffer (Clontech) at 45°C for two hours followed by four 20 min. washes in 2X SSC, 0.1% SDS at 45°C before phosphorimage analysis.

Hybridisations with the control oligonucleotide probes provides an estimate of the amount of DNA in the sample. As expected, none of the PCR

amplifications of BPH samples produced significantly detectable product, while 3 of 4 cancer samples gave a strong signal and one a very weak one.

Hybridisations with the conversion-specific probe showed a clear signal for the plasmid DNAs that matched the probe perfectly and for the 3 cancer samples for which there was stronger hybridisation with the control oligonucleotide probe. The fourth cancer sample that gave a very weak signal with the control oligonucleotide was barely detectable with the conversion-specific probe. This could have been due to the low level of DNA or, possibly, the presence of partially-converted DNA molecules. None of the plasmid clones that had mismatches to the conversion-specific probe gave a significant signal. The probe for unconverted DNA hybridised clearly with plasmid DNAs that had 0, 1 or 2 bases converted, but not with samples that had 8 or 10 converted bases. The hybridisations also indicated that there was a low level amplification of unconverted DNA in two BPH and one cancer sample (in this latter case there was a strong signal from probe for fully converted DNA, indicating that the PCR product was predominantly derived from properly converted DNA).

The results show that oligonucleotides of the type used here can discriminate between molecules that have been efficiently converted by bisulphite and those that have not. They can be used in a number of formats for detection of PCR products or prior to PCR or other detection methods to select out efficiently converted molecules of the target region from the total DNA population. The same approach can be used with primers that distinguish CpG methylated DNAs (or their derivatives containing C's) from unmethylated DNAs (containing U's or their derivatives containing T's).

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TABLE 1

	AATT	TTAA	ATAT	ACGT	CGCG	GATC	TCGA	AGAG
↓ o o o o	<i>Tsp509I</i>			<i>Mae II</i>		<u>Sau3A</u>		
↓ o o o o		<i>Mse I</i>					<i>Taq I</i>	
↓ o o o o					<i>BstUI</i>			
↓ o o o o				<i>Tai I</i>				
↓ A o o o o T	<i>Apo I</i>							
↓ A o o o o T		<i>Ase I</i>						
↓ A o o o o T			<i>Ssp I</i>					
↓ T o o o o A							<u>BstBI</u>	
↓ T o o o o A				<u>Sna BI</u>	<u>Nru I</u>			
↓ C o o o o G						<u>Pvu I</u>		
↓ G o o o o C	<u>EcoRI</u> <i>Apo I</i>							

TABLE 2.1 Primers for PCR Amplification of the Bisulphite-Modified GST-Pi Gene

PCR #	Target	Primer Name	Primer Type	Primer 5'	Primer 3'	Target size (bp)	Anneal °C	Genomic Position
-1	Upstream Top Strand DNA	GST-1	Outer	TTATGTAATAAAATTTCGTATATTTTCTATATG (SEQ ID NO: 23)		646	50/50	381-411
		GST-25	Inner	TCTAGATTATTTAAGTTTAGGAGTT (SEQ ID NO: 24)		499	50/50	495-519
		GST-3	Inner	AAACCTAAAAAATAAACAAACACAAA (SEQ ID NO: 25)		499	50/50	967-993
		GST-4	Outer	AAAAAACCTTCCCTCTTCCCAATCCC (SEQ ID NO: 26)		646	50/50	999-1027
1	Exon 1 Top Strand DNA	GST-9	Outer	TTTGTTGTTTGTATTATTTTACGTTT (SEQ ID NO: 27)		346	45/50	967-993
		GST-11	Inner	GGGATTTGGGAAAGAGGGAAGGTTT (SEQ ID NO: 28)		307	45/50	999-1025
		GST-12	Inner	ACTAAAACTCTAAACCCCATCCC (SEQ ID NO: 29)		307	45/50	1280-1303
		GST-10	Outer	AACCTAATACTACC TTAACCCGAT (SEQ ID NO: 30)		346	45/50	1304-1329

TABLE 2.1 continued

2	Exon 1 Bottom Strand DNA	GST-B1	Outer	AATGCTCTTCCTACTATCTATTTACTCGCTAAA (SEQ ID NO: 31)	387	50/55	958-990
		GST-B2	Inner	AAAACCTAAAAAATAAACTTCCC (SEQ ID NO: 32)	314	50/55	999-1027
		GST-B3	Inner	TGCGTTTATGTTGGGAGTTTGAAGTTT (SEQ ID NO: 33)	314	50/55	1285-1313
		GST-B4	Outer	TTTGTGGGAGTTGGGTTTGAATGTG (SEQ ID NO: 34)	387	50/55	1317-1345
3	Exon 2/Exon 3 Top Strand DNA	GST-13	Outer	GGTTAGAGTTTTAGTATGGGTTAAAT (SEQ ID NO: 35)	891	45/50	1287-1315
		GST-14	Inner	TAGTATTAGGTTAGGGTTTT (SEQ ID NO: 36)	803	45/50	1318-1337
		GST-15	Inner	AAGCTAACCCTAATCTACCAACAACATA (SEQ ID NO: 37)	803	45/50	1920-1892
		GST-16	Outer	CAAAAAAGCTTTAAATAAACCCCTCTACCA (SEQ ID NO: 38)	891	45/50	1978-1950

GGCTTT" 5'442960

TABLE 2.1 continued

4	Exon 5 Top Strand DNA	GST-30	Outer	GTTTTGTGGTTAGTTGTTTTTTAGGCTCTTAG (SEQ ID NO: 39)	340	55/60	2346-2376
		GST-31	Inner	GTTTTGAGTATTTTGTGTGCTAGTTTTT (SEQ ID NO: 40)	205	40/45	2381-2416
		GST-32	Inner	TTAATATAAATAAAAAAATATATTTACAA (SEQ ID NO: 41)	265	40/45	2617-2646
		GST-33	Outer	CAACCCCAATACCCAAACCTAATACAAATACTC (SEQ ID NO: 42)	340	55/60	2653-2686
5	Exon 7 Top Strand DNA	GST-26	Outer	GGTTTTAGTTTTTGGTTGTTGGATG (SEQ ID NO: 43)	347	50/55	3845-3869
		GST-27	Inner	TTTTTTGTTTTTAGTATATGCGGG (SEQ ID NO: 44)	287	50/55	3874-3899
		GST-28	Inner	ATACTAAAAAACTATTTTCTAATCCTCTA (SEQ ID NO: 45)	287	50/55	4161-4132
		GST-29	Outer	CCAAACTAAAAACTGCCAAAAAACCACTAA (SEQ ID NO: 46)	347	50/55	4192-4164

Bases arising due to C to U conversion by bisulphite treatment are shown in bold

TABLE 2.2 Primers for Direct Sequencing of Amplified GST-Pi Gene PCR Fragments

PCR #	Target	Primer Name	Primer Type	Primer 5' 3'	Target size (bp)	Anneal °C	Genomic Position
1	Exon 1 Top Strand DNA	GST-11	M13	TGTAAGACGACGGCCAGTGGGATTGGGAAAGCGGAA (SEQ ID NO: 47)	307	45/50	1003-1026
		GST-12	Biotin	BioACTAAAAAAGTCTAAACCCCATCCC	307	45/50	1286-1313
2	Exon 1 Bottom Strand DNA	GST-B2	M13	TGTAAGACGACGGCCAGTGTGGGAGTTTCAGTTTT (SEQ ID NO: 48)	314	50/55	999-1027
		GST-B2	Biotin	BioAAAAAGCTAAAAAAGAAAAAAGTTCCC (314	50/55	1286-1313

TABLE 2.2 continued

3	Exon 2/3 Top Strand DNA	GST-14	M13	<u>TGTAAACGACGCGCAGTTAGTATTAGCTTA</u> (SEQ ID NO: 49)	603	45/50	1317-1337
		GST-15	Biotin	BioAACTCTTAACCCCTAACTACCAACAACATA	603	45/50	1920-1892
4	Exon 4/5 Top Strand DNA	GST-31	M13	<u>TGTAAACGACGCGCAGTTTTCAGTATTGTTGTG</u> (SEQ ID NO: 50)	265	55/60	2381-2410
		GST-32	Biotin	BioTTAATATAAATAAAAAAATATATTTTACAA	265	55/60	2617-2646
5	Exon 7 Top Strand DNA	GST-27	M13	<u>TGTAAACGACGCGCAGTCTTTTAGTATATGTGG</u> (SEQ ID NO: 51)	287	50/55	3874-4132
		GST-28	Biotin	BioATACTAAAAAAGCTATTTTCTAATCGCTCTA	287	50/55	4161-4164

Extensions on "M13" primers for annealing primer is underlined.

TABLE 3

Primer	Forward or Reverse	Primer Sequence (5'-3')	Co-ordinates	CpG sites
CGPS-1	F	CGCGAGGTTTCGTGGAGTTTCGTGTC (SEQ ID NO: 1)	1210-1238	-3 to +3
CGPS-2	F	CGTATTAGTAGTAGCGGGTTC (SEQ ID NO: 2)	1247-1271	+4 to +8
CGPS-3	R	TCCGATCCTCCCGGAAACGCTCG (SEQ ID NO: 8)	1428-1452	+21 to +23
CGPS-4	R	GAACGCTCGAACCCCTAAAAACGCTAACG (SEQ ID NO: 9)	1406-1438	+19 to +23
CGPS-5	F	YGGTTTAGGGAATTTTTCGG (SEQ ID NO: 3)	894-917	-39 to -37
CGPS-6	F	YGGYGTAGTGTGTTGTTATATTC (SEQ ID NO: 4)	925-952	-36 to -31
CGPS-11	F	GGGAATTTTTCGGGATGTTTGGCGC (SEQ ID NO: 5)	902-930	-38 to -34
CGPS-7	R	CRGCTAAATCCCRAAATCCCGCG (SEQ ID NO: 10)	1038-1064	-23 to -27
CGPS-8	R	ACCCRACRACCTACACCCRAACGTCG (SEQ ID NO: 11)	1077-1106	-16 to -21
CGPS-9	R	CTCTTCTAAATAATCCCGGAAATCGCG (SEQ ID NO: 12)	1113-1143	-12 to -15
CGPS-12	R	AAACRCCCTAAATCCCGGAAATCGCG (SEQ ID NO: 13)	1040-1068	-23 to -26
CGPS-13	R	AACTCCRCGACCCGACCCGAGACCG (SEQ ID NO: 14)	1094-1123	-14 to -18
CGPS-21	F	TTTTAGGGGTTTGGAGGTTTC (SEQ ID NO: 6)	1415-1438	+21 to +23
CGPS-22	F	GGTAGGTTCTGTTATCGC (SEQ ID NO: 7)	1473-1491	+26 to +28
CGPS-23	R	AAAAATCRAATCTCTCCGAATAAACG (SEQ ID NO: 15)	1640-1666	+36 to +34
CGPS-24	R	AAAAACCAAATAAAACACACGACG (SEQ ID NO: 16)	1676-1703	+39 to +37

00/2217 8thE/960

TABLE 4

	Assay negative	Assay positive
Normal subjects	10	0
Benign hyperplasia	17	1
Cancer (total)	7	17
Stage A	1	3
Stage B	3	3
Stage C	0	2
Stage D	0	7
Stage not defined	3	2

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Claims:

1. A diagnostic or prognostic assay for a disease or condition in a subject,
said disease or condition characterised by abnormal methylation of cytosine
at a site or sites within the glutathione-S-transferase (GST) Pi gene and/or its
regulatory flanking sequences, wherein said assay comprises the steps of;

(i) isolating DNA from said subject,

(ii) exposing said isolated DNA to reactants and conditions for the
amplification of a target region of the GST-Pi gene and/or its regulatory
flanking sequences which includes a site or sites at which abnormal cytosine
methylation characteristic of the disease or condition occurs, the
amplification being selective in that it only amplifies the target region if the
said site or sites at which abnormal cytosine methylation occurs is/are
methylated, and

(iii) determining the presence of amplified DNA,
wherein the amplifying step (ii) is used to amplify a target region within the
region of the GST-Pi gene and/or its regulatory flanking sequences defined by
(and inclusive of) CpG sites -43 to +55.

2. An assay according to claim 1, wherein prior to the amplifying step,
the isolated DNA is treated such that unmethylated cytosines are converted
to uracil or another nucleotide capable of forming a base pair with adenine
while methylated cytosines are unchanged or are converted to a nucleotide
capable of forming a base pair with guanine.

3. As assay according to any one of the preceding claims, wherein the
amplifying step involves polymerase chain reaction (PCR) amplification.

4. An assay according to claim 3, wherein said PCR amplification utilises
a reverse primer including guanine at at least one site whereby, upon the

reverse primer annealing to the treated DNA, said guanine will either form a base pair with a methylated cytosine (or another nucleotide to which the methylated cytosine has been converted through said treatment) if present, or will form a mismatch with uracil (or another nucleotide to which unmethylated cytosine has been converted through said treatment).

5. An assay according to claim 4, wherein said PCR amplification utilises a forward primer including cytosine at at least one site(s) corresponding to cytosine nucleotides that are abnormally methylated in the DNA of a subject with the disease or condition being assayed.

6. An assay according to claim 5, wherein the primers are of 12 to 30 nucleotides in length.

7. An assay according to claim 6, wherein the primers are selected so as to anneal to a sequence within the target region that includes two to four cytosine nucleotides that are abnormally methylated in the isolated DNA of a subject with the disease or condition being assayed.

8. An assay according to claim 2, wherein the treatment of the isolated DNA involves reacting the isolated DNA with bisulphite.

9. An assay according to claim 8, wherein the amplifying step involves polymerase chain reaction (PCR) amplification.

10. An assay according to claim 9, wherein said PCR amplification utilises a reverse primer including guanine at at least one site whereby, upon the reverse primer annealing to the treated DNA, said guanine will either form a base pair with a methylated cytosine if present, or will form a mismatch with uracil.

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11. An assay according to claim 10, wherein said PCR amplification
utilises a forward primer including cytosine at at least one site(s)
corresponding to cytosine nucleotides that are abnormally methylated in the
isolated DNA of a subject with the disease or condition being assayed.

12. An assay according to claim 11, wherein the primers are of 12 to 30
nucleotides in length.

13. An assay according to claim 12, wherein the primers are selected so as
to anneal to a sequence within the target region that includes two to four
cytosine nucleotides that are abnormally methylated in the DNA of a subject
with the disease or condition being assayed.

14. An assay according to any one of the preceding claims, wherein said
DNA is isolated from cells from tissue, blood (including serum and plasma),
semen, urine, lymph or bone marrow.

15. An assay according to any one of the preceding claims, wherein the
disease or condition to be assayed is selected from cancers.

16. An assay according to claim 15, wherein the disease or condition to be
assayed is selected from prostate cancer, breast cancer, cervical cancer and
liver cancer.

17. An assay according to claim 16, wherein the disease or condition to be
assayed is prostate cancer.

18. An assay according to claim 17, wherein the amplifying step is used to
amplify a target region within the region of the GST-Pi gene and its

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regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +53.

19. An assay according to claim 17, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +10.

20. An assay according to claim 17, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to -14.

21. An assay according to claim 17, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to -8.

22. An assay according to any one of the preceding claims wherein the target region excludes any or all of the CpG sites -36, -32, -23, -20, -19 and -14.

23. An assay according to any one of claims 5 to 21, wherein if either or both of the reverse or forward primers anneal to a sequence within the target region that includes any or all of CpG sites -36, -32, -23, -20, -19 and -14, then said PCR amplification further utilises equivalent reverse and/or forward primers including a redundant nucleotide(s) at the position(s) within their sequence(s) corresponding to cytosine or methylated cytosine of CpG sites -36, -32, -23, -20, -19 and -14.

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24. An assay according to claim 17, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites +9 to +53.

5

25. An assay according to claim 17, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites +1 to +53.

10

26. An assay according to claim 17, wherein the amplifying step involves PCR amplification using primer pairs consisting of a forward and reverse primer selected from each of the following groups:

Forward Primers

15

CGCGAGGTTTTTCGTTGGAGTTTCGTCGTC	(SEQ ID NO: 1)
CGTTATTAGTGAGTACGCGCGGTTTC	(SEQ ID NO: 2)
YGGTTTTAGGGAATTTTTTTTCGC	(SEQ ID NO: 3)
YGGYGYGTTAGTTYGTTGYGTATATTTTC	(SEQ ID NO: 4)
GGGAATTTTTTTTCGCGATGTTYGGCGC	(SEQ ID NO: 5)
TTTTTAGGGGGTTYGGAGCGTTTC	(SEQ ID NO: 6)
GGTAGGTTGYGTTTATCGC	(SEQ ID NO: 7)

20

Reverse Primers

25

TCCCATCCCTCCCGAAACGCTCCG	(SEQ ID NO: 8)
GAAACGCTCCGAACCCCTAAAAACCGCTAACG	(SEQ ID NO: 9)
CRCCCTAAAATCCCRAAATCRCCGCG	(SEQ ID NO: 10)
ACCCCRACRACRCTACACCCRAACGTGG	(SEQ ID NO: 11)
CTCTTCTAAAAAATCCCRCAACTCCCGCCG	(SEQ ID NO: 12)
AAAACRCCCTAAAATCCCGGAAATCGCCG	(SEQ ID NO: 13)
AACTCCRCGACCCCAACCCGACGACCG	(SEQ ID NO: 14)
AAAAATTCTAATCTCTCCGAATAAACG	(SEQ ID NO: 15)

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AAAAACCRAAATAAAAACCACACGACG (SEQ ID NO: 16),

wherein Y is C, T or a mixture thereof, and R is A, G or a mixture thereof.

27. An assay according to claim 17, wherein the amplifying step involves
5 PCR amplification using primer pairs consisting of a forward and reverse
primer selected from each of the following groups:

Forward Primers

CGCGAGGTTTTCGTTGGAGTTTCGTCTGTC (SEQ ID NO: 1)

CGTTATTAGTGAGTACGCGCGGTTC (SEQ ID NO: 2)

Reverse Primers

TCCCATCCCTCCCGAAACGCTCCG (SEQ ID NO: 8)

GAAACGCTCCGAACCCCTAAAAACCGCTAACG (SEQ ID NO: 9).

28. An assay according to claim 17, wherein the amplifying step involves
15 PCR amplification using primer pairs consisting of a forward and reverse
primer selected from each of the following groups:

Forward Primers

YGGTTTTAGGGAATTTTTTTTCGC (SEQ ID NO: 3)

YGGYGYGTTAGTTYGTTGYGTATATTTTC (SEQ ID NO: 4)

GGGAATTTTTTTTCGCGATGTTTYGGCGC (SEQ ID NO: 5)

Reverse Primers

CRCCCTAAAAATCCCCRAAATCRCCGCG (SEQ ID NO: 10)

ACCCCRACRACCRCTACACCCRAACGTCG (SEQ ID NO: 11)

CTCTTCTAAAAAATCCCRGRAACTCCGCGCG (SEQ ID NO: 12)

AAAACRCCCTAAAAATCCCCGAAATCGCCG (SEQ ID NO: 13)

AACTCCCRCCGACCCCAACCCGACGACCG (SEQ ID NO: 14),

wherein Y is C, T or a mixture thereof and R is A, G or a mixture thereof.

29. An assay according to claim 17, wherein the amplifying step involves
30 PCR amplification using primer pairs consisting of a forward and reverse

primer selected from each of the following groups:

Forward Primers

TTTTTAGGGGGTTYGGAGCGTTTC (SEQ ID NO: 6)

GGTAGGTTGYGTTTATCGC (SEQ ID NO: 7)

Reverse Primers

AAAAATTCTAATCTCTCCGAATAAACG (SEQ ID NO: 15)

AAAAACCRAAATAAAAACCACACGACG (SEQ ID NO: 16),

wherein Y is C, T or a mixture thereof, and R is A, G or a mixture thereof.

30. An assay according to claim 16, wherein the disease or condition to be assayed is liver cancer.

31. An assay according to claim 30, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to -14.

32. An assay according to claim 31, wherein the target region excludes any or all of the CpG sites -36, -32, -23, -20, -19, and -14.

33. An assay according to claim 30, wherein if either or both of the reverse or forward primers anneal to a sequence within the target region that includes any or all of CpG sites -36, -32, -23, -20, -19 and -14, then said PCR amplification further utilises equivalent reverse and/or forward primers including a redundant nucleotide(s) at the position(s) within their sequence(s) corresponding to cytosine or methylated cytosine of CpG sites -36, -32, -23, -20, -19 and -14.

34. An assay according to claim 30, wherein the amplifying step is used to amplify a target region within the region of the GST-Pi gene and its

regulatory flanking sequences defined by (and inclusive of) CpG sites +9 to +53.

35. A diagnostic or prognostic assay for a disease or condition in a subject said disease or condition characterised by abnormal methylation of cytosine at a site or sites within the glutathione-S-transferase (GST) Pi gene and/or its regulatory flanking sequences, wherein said assay comprises the steps of;

- (i) isolating DNA from said subject, and
- (ii) determining the presence of abnormal methylation of cytosine at a site or sites within the region of the GST-Pi gene and/or its regulatory flanking sequences defined by (and inclusive of) CpG sites -43 to +55.

36. An assay according to claim 35, wherein the region of the GST-Pi gene and its regulatory flanking sequences within which the presence of methylated cytosine(s) at a site or sites is determined is selected from the regions defined by (and inclusive of) CpG sites -43 to +53, -43 to +10, -43 to -14, +9 to +53 and +1 to +53.

37. An assay according to claim 35 or 36, wherein the said region of the GST-Pi gene and its regulatory flanking sequences excludes any or all of the CpG sites -36, -32, -23, -20, -19 and -14.

38. An assay according to claim 36, wherein the region of the GST-Pi gene and its regulatory flanking sequences within which the presence of methylated cytosine(s) at a site or sites is determined is the region defined by (and inclusive of) CpG sites +9 to +53.

39. An assay according to claim 36, wherein the region of the GST-Pi gene and its regulatory flanking sequences within which the presence of

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methyated cytosine(s) at a site or sites is determined is the region defined by (and inclusive of) CpG sites +1 to +53.

40. An assay according to any one of claims 35 to 39, wherein prior to the determination step, the isolated DNA is treated such that unmethylated cytosines are converted to uracil or another nucleotide capable of forming a base pair with adenine while methylated cytosines are unchanged or are converted to a nucleotide capable of forming a base pair with guanine.

41. An assay according to claim 40, wherein the treatment of the isolated DNA involves reacting the isolated DNA with bisulphite.

42. An assay according to any one of claims 35 to 41, wherein the determination step involves selective hybridisation of oligonucleotide/polynucleotide/peptide-nucleic acid (PNA) probe(s).

43. An assay according to claim 42, wherein if the probe(s) hybridise to a sequence within the target region that includes any or all of CpG sites -36, -32, -23, -20, -19 and -14, then said selective hybridisations further utilises equivalent probe(s) including a redundant nucleotide(s) at the position(s) within their sequence(s) corresponding to cytosine or methylated cytosine of CpG sites -36, -32, -23, -20, -19 and -14.

44. An assay according to any one of claims 35 to 43, wherein said DNA is isolated from cells from tissue, blood (including serum and plasma), semen, urine, lymph or bone marrow.

45. An assay according to any one of claims 35 to 43, wherein the disease or condition to be assayed is selected from cancers.

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46. An assay according to claim 45, wherein the disease or condition to be assayed is selected from prostate cancer, breast cancer, cervical cancer and liver cancer.

5 47. An assay according to claim 46, wherein the disease or condition to be assayed is prostate cancer.

48. An assay according to claim 46, wherein the disease or condition to be assayed is liver cancer.

10 49. A primer or probe comprising a nucleotide sequence selected from the group consisting of:

CGCGAGGTTTTCGTTGGAGTTTCGTCGTC	(SEQ ID NO: 1)
CGTTATTAGTGAGTACGCCGCGTTTC	(SEQ ID NO: 2)
15 YGGTTTTAGGGAATTTTTTTTCGC	(SEQ ID NO: 3)
YGGYGYGTTAGTTYGTTGYGTATATTTTC	(SEQ ID NO: 4)
GGGAATTTTTTTTCGCGATGTTTTYGGCGC	(SEQ ID NO: 5)
TTTTTAGGGGGTTTGGAGCGTTTC	(SEQ ID NO: 6)
GGTAGGTTGYGTTTATCGC	(SEQ ID NO: 7)
20 AAAAAATTCRAATCTCTCCGAATAAACG	(SEQ ID NO: 8)
AAAAACCRAATAAAAAACACACGACG	(SEQ ID NO: 9)
TCCCATCCCTCCCGAAACGCTCCG	(SEQ ID NO: 10)
GAAACGCTCCGAACCCCTAAAAACCGCTAACG	(SEQ ID NO: 11)
CRCCTAAAAATCCCRRAATCRCCGCG	(SEQ ID NO: 12)
25 ACCCRACRACRCTACACCCRAACGTCG	(SEQ ID NO: 13)
CTCTTCTAAAAAATCCCRRAACTCCGCGC	(SEQ ID NO: 14)
AAAACRCCCTAAAAATCCCGAAATCGCCG	(SEQ ID NO: 15)
AACTCCCRCCGACCCCAACCCGACGACCG,	(SEQ ID NO: 16),

wherein Y is a mixture of C and T, and R is a mixture of A and G.

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50. A probe comprising a nucleotide sequence selected from the group consisting of:

AAACCTAAAAAATAAACAAACAA (SEQ ID NO: 17)

GGGCCTAGGGAGTAAACAGACAG (SEQ ID NO: 18)

5 CCTTTCCCTCTTTCCCAARRTCCCCA (SEQ ID NO: 19)

TTTGGTATTTTPTTCGGGTTTTAG (SEQ ID NO: 20)

CTTGGCATCCTCCCCCGGGCTCCAG (SEQ ID NO: 21)

GGYAGGGAAGGGAGGYAGGGGYTGGG (SEQ ID NO: 22).

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1/17

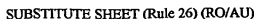


Figure 2
Upstream Region of Differential Methylation in Prostate Cancer

[illegible]

TC

3/17

Figure 2 (Continued)

-15 -14 -13-12 -11 -10 -9 -8 -7 -6 -5
 GCCGCGGGA GTCCGCGGA CCTCCAGAA GAGCGCCGG CCCCCTGACT CAGCACTGGG GCGGAGCGG GCGGGACCAC -35
 GTTGTGGGA GTTGTGGGA TTTTITAGAA GAGTGGTGG GAGTGTGATT TAGTATTGGG GTGAGTGGG GTGGGATTAT B-U
 GTCGCGGGA GTTCGCGGA TTTTITAGAA GAGCGGTGG CAGTGTGATT TAGTATTGGG GCGGAGCGG GCGGGATTAT B-M

 <GCCGCCCT CAARCRCCCT AAAAAATCTT CTC CGPS-9
 CAGCCRCCT CAA CGPS-13

 -4 -3 -2 -1 > 1 2 3 4 5 6 7
 CCTTATAAGG CTCGAGGCC GCGAGGCCCTT CGCTGGAGTT TCGCGCGGC AGTCTTCCG ACCAGTGAAT ACGCGGGCC +46
 TTTTATAAGG TTTCGAGGTT GTGAGGTTTT TGTTCGAGTT TGTTCGAGTT AGTTTGTGT ATTAGTGAAT ATGTGTGTT B-U
 TTTTATAAGG TTTCGAGGTT GCGAGGTTTT CGTTGGAGTT TCGTGTGCT AGTTTGTGT ATTAGTGAAT ACGCGGGTT B-M

 CGPS-1 C GCGAGGTTTT CGTTGGAGTT TGTGTGTC> CGPS-2 CGTT ATTAGTGAAT ACGCGGGTT

 8 9 10
 CGCGTCCCG GGGATGGGC TCAGAGCTCC CAGCATGGG CCAA +90
 TGTGTTTTG GGGATGGGT TTAGAGTTTT TAGTATGGG TTAA B-U
 CGCGTTTCG GGGATGGGT TTAGAGTTTT TAGTATGGG TTAA B-M

 C>

Figure 3A Methylation Status of Individual Sites in the GST-Pi Gene

site	LN	Dn	PC3 M	PC3 MM	PC3 CN	2A	2B	2C	3A	3B	3C	4A	4B	4C	5A	5B	5C	6A	6B	6C	7A	7B	7C	8A	8B	8C	9A	9B	9C	10A	10B	10C	11A	11B	11C	12A	12B	12C	13A	13B	13C	14A	14B	14C	15A	15B	15C	16A	16B	16C	17A	17B	17C	18A	18B	18C	19A	19B	19C	20A	20B	20C	21A	21B	21C	22A	22B	22C	23A	23B	23C	24A	24B	24C	25A	25B	25C	26A	26B	26C	27A	27B	27C	28A	28B	28C	29A	29B	29C	30A	30B	30C	31A	31B	31C	32A	32B	32C	33A	33B	33C	34A	34B	34C	35A	35B	35C	36A	36B	36C	37A	37B	37C	38A	38B	38C	39A	39B	39C	40A	40B	40C	41A	41B	41C	42A	42B	42C	43A	43B	43C	44A	44B	44C	45A	45B	45C	46A	46B	46C	47A	47B	47C	48A	48B	48C	49A	49B	49C	50A	50B	50C	51A	51B	51C	52A	52B	52C	53A	53B	53C	54A	54B	54C	55A	55B	55C	56A	56B	56C	57A	57B	57C	58A	58B	58C	59A	59B	59C	60A	60B	60C	61A	61B	61C	62A	62B	62C	63A	63B	63C	64A	64B	64C	65A	65B	65C	66A	66B	66C	67A	67B	67C	68A	68B	68C	69A	69B	69C	70A	70B	70C	71A	71B	71C	72A	72B	72C	73A	73B	73C	74A	74B	74C	75A	75B	75C	76A	76B	76C	77A	77B	77C	78A	78B	78C	79A	79B	79C	80A	80B	80C	81A	81B	81C	82A	82B	82C	83A	83B	83C	84A	84B	84C	85A	85B	85C	86A	86B	86C	87A	87B	87C	88A	88B	88C	89A	89B	89C	90A	90B	90C	91A	91B	91C	92A	92B	92C	93A	93B	93C	94A	94B	94C	95A	95B	95C	96A	96B	96C	97A	97B	97C	98A	98B	98C	99A	99B	99C	100A	100B	100C	101A	101B	101C	102A	102B	102C	103A	103B	103C	104A	104B	104C	105A	105B	105C	106A	106B	106C	107A	107B	107C	108A	108B	108C	109A	109B	109C	110A	110B	110C	111A	111B	111C	112A	112B	112C	113A	113B	113C	114A	114B	114C	115A	115B	115C	116A	116B	116C	117A	117B	117C	118A	118B	118C	119A	119B	119C	120A	120B	120C	121A	121B	121C	122A	122B	122C	123A	123B	123C	124A	124B	124C	125A	125B	125C	126A	126B	126C	127A	127B	127C	128A	128B	128C	129A	129B	129C	130A	130B	130C	131A	131B	131C	132A	132B	132C	133A	133B	133C	134A	134B	134C	135A	135B	135C	136A	136B	136C	137A	137B	137C	138A	138B	138C	139A	139B	139C	140A	140B	140C	141A	141B	141C	142A	142B	142C	143A	143B	143C	144A	144B	144C	145A	145B	145C	146A	146B	146C	147A	147B	147C	148A	148B	148C	149A	149B	149C	150A	150B	150C	151A	151B	151C	152A	152B	152C	153A	153B	153C	154A	154B	154C	155A	155B	155C	156A	156B	156C	157A	157B	157C	158A	158B	158C	159A	159B	159C	160A	160B	160C	161A	161B	161C	162A	162B	162C	163A	163B	163C	164A	164B	164C	165A	165B	165C	166A	166B	166C	167A	167B	167C	168A	168B	168C	169A	169B	169C	170A	170B	170C	171A	171B	171C	172A	172B	172C	173A	173B	173C	174A	174B	174C	175A	175B	175C	176A	176B	176C	177A	177B	177C	178A	178B	178C	179A	179B	179C	180A	180B	180C	181A	181B	181C	182A	182B	182C	183A	183B	183C	184A	184B	184C	185A	185B	185C	186A	186B	186C	187A	187B	187C	188A	188B	188C	189A	189B	189C	190A	190B	190C	191A	191B	191C	192A	192B	192C	193A	193B	193C	194A	194B	194C	195A	195B	195C	196A	196B	196C	197A	197B	197C	198A	198B	198C	199A	199B	199C	200A	200B	200C	201A	201B	201C	202A	202B	202C	203A	203B	203C	204A	204B	204C	205A	205B	205C	206A	206B	206C	207A	207B	207C	208A	208B	208C	209A	209B	209C	210A	210B	210C	211A	211B	211C	212A	212B	212C	213A	213B	213C	214A	214B	214C	215A	215B	215C	216A	216B	216C	217A	217B	217C	218A	218B	218C	219A	219B	219C	220A	220B	220C	221A	221B	221C	222A	222B	222C	223A	223B	223C	224A	224B	224C	225A	225B	225C	226A	226B	226C	227A	227B	227C	228A	228B	228C	229A	229B	229C	230A	230B	230C	231A	231B	231C	232A	232B	232C	233A	233B	233C	234A	234B	234C	235A	235B	235C	236A	236B	236C	237A	237B	237C	238A	238B	238C	239A	239B	239C	240A	240B	240C	241A	241B	241C	242A	242B	242C	243A	243B	243C	244A	244B	244C	245A	245B	245C	246A	246B	246C	247A	247B	247C	248A	248B	248C	249A	249B	249C	250A	250B	250C	251A	251B	251C	252A	252B	252C	253A	253B	253C	254A	254B	254C	255A	255B	255C	256A	256B	256C	257A	257B	257C	258A	258B	258C	259A	259B	259C	260A	260B	260C	261A	261B	261C	262A	262B	262C	263A	263B	263C	264A	264B	264C	265A	265B	265C	266A	266B	266C	267A	267B	267C	268A	268B	268C	269A	269B	269C	270A	270B	270C	271A	271B	271C	272A	272B	272C	273A	273B	273C	274A	274B	274C	275A	275B	275C	276A	276B	276C	277A	277B	277C	278A	278B	278C	279A	279B	279C	280A	280B	280C	281A	281B	281C	282A	282B	282C	283A	283B	283C	284A	284B	284C	285A	285B	285C	286A	286B	286C	287A	287B	287C	288A	288B	288C	289A	289B	289C	290A	290B	290C	291A	291B	291C	292A	292B	292C	293A	293B	293C	294A	294B	294C	295A	295B	295C	296A	296B	296C	297A	297B	297C	298A	298B	298C	299A	299B	299C	300A	300B	300C	301A	301B	301C	302A	302B	302C	303A	303B	303C	304A	304B	304C	305A	305B	305C	306A	306B	306C	307A	307B	307C	308A	308B	308C	309A	309B	309C	310A	310B	310C	311A	311B	311C	312A	312B	312C	313A	313B	313C	314A	314B	314C	315A	315B	315C	316A	316B	316C	317A	317B	317C	318A	318B	318C	319A	319B	319C	320A	320B	320C	321A	321B	321C	322A	322B	322C	323A	323B	323C	324A	324B	324C	325A	325B	325C	326A	326B	326C	327A	327B	327C	328A	328B	328C	329A	329B	329C	330A	330B	330C	331A	331B	331C	332A	332B	332C	333A	333B	333C	334A	334B	334C	335A	335B	335C	336A	336B	336C	337A	337B	337C	338A	338B	338C	339A	339B	339C	340A	340B	340C	341A	341B	341C	342A	342B	342C	343A	343B	343C	344A	344B	344C	345A	345B	345C	346A	346B	346C	347A	347B	347C	348A	348B	348C	349A	349B	349C	350A	350B	350C	351A	351B	351C	352A	352B	352C	353A	353B	353C	354A	354B	354C	355A	355B	355C	356A	356B	356C	357A	357B	357C	358A	358B	358C	359A	359B	359C	360A	360B	360C	361A	361B	361C	362A	362B	362C	363A	363B	363C	364A	364B	364C	365A	365B	365C	366A	366B	366C	367A	367B	367C	368A	368B	368C	369A	369B	369C	370A	370B	370C	371A	371B	371C	372A	372B	372C	373A	373B	373C	374A	374B	374C	375A	375B	375C	376A	376B	376C	377A	377B	377C	378A	378B	378C	379A	379B	379C	380A	380B	380C	381A	381B	381C	382A	382B	382C	383A	383B	383C	384A	384B	384C	385A	385B	385C	386A	386B	386C	387A	387B	387C	388A	388B	388C	389A	389B	389C	390A	390B	390C	391A	391B	391C	392A	392B	392C	393A	393B	393C	394A	394B	394C	395A	395B	395C	396A	396B	396C	397A	397B	397C	398A	398B	398C	399A	399B	399C	400A	400B	400C	401A	401B	401C	402A	402B	402C	403A	403B	403C	404A	404B	404C	405A	405B	405C	406A	406B	406C	407A	407B	407C	408A	408B	408C	409A	409B	409C	410A	410B	410C	411A	411B	411C	412A	412B	412C	413A	413B	413C	414A	414B	414C	415A	415B	415C	416A	416B	416C	417A	417B	417C	418A	418B	418C	419A	419B	419C	420A	420B	420C	421A	421B	421C	422A	422B	422C	423A	423B	423C	424A	424B	424C	425A	425B	425C	426A	426B	426C	427A	427B	427C	428A	428B	428C	429A	429B	429C	430A	430B	430C	431A	431B	431C	432A	432B	432C	433A	433B	433C	434A	434B	434C	435A	435B	435C	436A	436B	436C	437A	437B	437C	438A	438B	438C	439A	439B	439C	440A	440B	440C	441A	441B	441C	442A	442B	442C	443A	443B	443C	444A	444B	444C	445A	445B	445C	446A	446B	446C	447A	447B	447C	448A	448B	448C	449A	449B	449C	450A	450B	450C	451A	451B	451C	452A	452B	452C	453A	453B	453C	454A	454B	454C	455A	455B	455C	456A	456B	456C	457A	457B	457C	458A	458B	458C	459A	459B	459C	460A	460B	460C	461A
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Figure 3A (cont'd)

1	++++	-	++	B	B	-	-	++	++	++	++++	+++	-
2	++++	-	++	B	B	-	-	++	++	++	++++	+++	-
3	++++	-	++	B	B	-	-	++	++	++	++++	+++	-
4	++++	-	++	B	B	-	-	++	++	++	++++	+++	-
5	++++	-	+++	B	B	-	-	B	++	+++	++++	++++	-
6	++++	-	+++	B	B	-	-	B	+++	+++	++++	++++	-
7	++++	-	+++	B	B	-	-	B	+++	+++	++++	+++	-
8	++++	-	++	B	B	-	-	B	++	++	++++	+++	-
9	++++	-	++	B	B	-	-	B	++	++	++++	+++	-
10	++++	-	++	B	B	-	-	B	++	++	++++	+++	-
11													
12													
13	++++		+	+++	+++		-	+++	+++	+++	++++	+++	
14	++++		+	+++	+++		-	+++	+++	+++	++++	+++	
15	++++		+	+++	+++		-	+++	+++	+++	++++	+++	
16	++++		+	+++	+++		-	+++	+++	+++	++++	+++	
17	++++		+	+++	+++		-	+++	+++	+++	++++	+++	
18	++++		+	++	+		-	++	+++	+++	++++	+++	
19	++++		+	+	+		-	++	+++	+++	++++	+++	
20	++++		B	+	+		-	+++	+++	+++	++++	+++	
21	++++		B	+	+		-	+++	+++	+++	++++	+++	
22	++++		B	+	+		-	+++	+++	+++	++++	+++	
23	++++		B	+	+		-	+++	+++	+++	++++	+++	
24	++++		B	+	+		-	+++	+++	+++	++++	+++	
25	+		B	+	+		-	+++	+++	+++	++++	+++	
26	++++		B	++	B		-	+++	+++	+++	++++	+++	
27	++++		B	+++	B		-	+++	+++	+++	++++	+++	
28	++++		B	+++	B		-	+++	+++	+++	++++	+++	
29	++++		B	+++	B		-	B	+++	+++	++++	+++	
30	++++		B	B	B		-	+++	B	+++	++++	+++	
31	++++		B	B	B		-	+++	B	+++	++++	+++	
32	B		B	B	B		-	+++	B	+++	++++	+++	
33	++++		B	+++	B		-	+++	+++	+++	++++	+++	

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Figure 3B Methylation Status of Individual Sites in the GST-P1 Gene

Site	NormalP rostate (15)	PC-3 (10)	LNcaP (7)	BC (9)	CC (4)	DC (10)	Blood (13)	Brain (6)	Spleen (6)	Liver (6)	Smooth muscle (6)	Lung (5)	Bone marrow (5)	Pancreas (6)	Heart (3)
56	+++	+++	++++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++
55	++++	+	++++	++	+++	+++	+++	+++	+++	++	+++	++	+++	+++	+++
54	++	-	++++	+++	+	+++	+++	+++	+++	++	+++	+++	+++	++	+++
53	+++	-	++++	+	+	+++	+++	+++	+++	+	-	+	+++	++	+++
52	++	+	++++	+	+	+++	+++	+++	+++	+	+	+++	+++	++	+++
51	+	-	++++	++	++	+++	+++	+++	+++	++	+	+++	+++	++	+++
49	++	++	++++	++	++	+++	+++	+++	+++	++	+	+++	+++	++	+++
48	++	+	++++	+	B	+++	+++	+++	+++	++	+	+++	+++	++	+++
47	++	+++	++++	++	++	+++	+++	+++	+++	+	-	+	+++	-	+++
46	+	++	++++	+++	++	+++	+++	+++	+++	+	-	+++	+++	+	+
45	++	-	++++	+++	++	+++	+++	+++	+++	+	-	+++	+++	++	+
44	++	-	++++	+	++	+++	+++	+++	+++	+	-	+++	+++	+	+
43	+	-	++++	+	+++	+++	+++	+++	+++	+	-	+	+	++	+++
42	-	-	++++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-
41	-	-	++++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-
40	-	-	++++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-
39	-	-	++++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-
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35	+	+	++++	+++	+	+++	+++	+++	+++	-	-	-	-	-	-
34	-	-	++++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-
33	-	p	++++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-
32	-	B	+++	+++	+++	+++	+++	+++	+++	++	-	-	+	-	-
31	-	B	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-
30	-	B	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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PCT/AU99/00306
Received 30 June 1999

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FIGURE 3B (cont'd)

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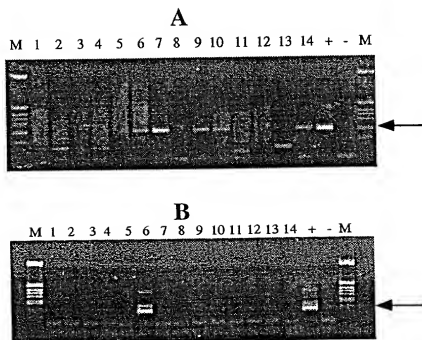


FIGURE 4A

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Sample Number

1 2 3 4 5 6 7 8 9 10

M n c n c n c n c n c n c n c n c + -



Sample	Tissue	Gleason	% Methylation Non CG rich PCR
1	Normal	N/A	-
	Cancer	3+3	++++
2	Normal	N/A	-
	Cancer	3+5	++
3	Normal	N/A	-
	Cancer	3+3	++
4	Normal	N/A	-
	Cancer	3+5	-
5	Normal	N/A	-
	Cancer	2+2	++
6	Normal	N/A	-
	Cancer	3+3	-
7	Normal	N/A	-
	Cancer	2+3	++
8	Normal	N/A	-
	Cancer	3+3	++
9	Normal	N/A	-
	Cancer	2+3	++++
10	Normal	N/A	-
	Cancer	?	++

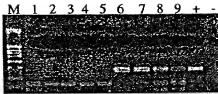
FIGURE 4B

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A



B



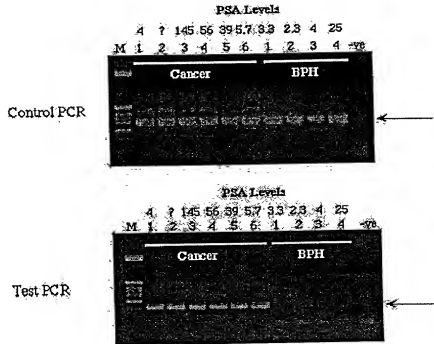
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FIGURE 4C

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Figure 6



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Figure 7A

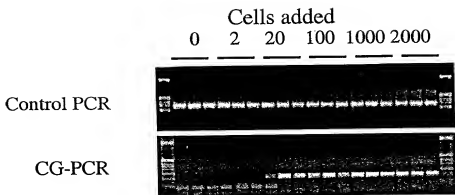
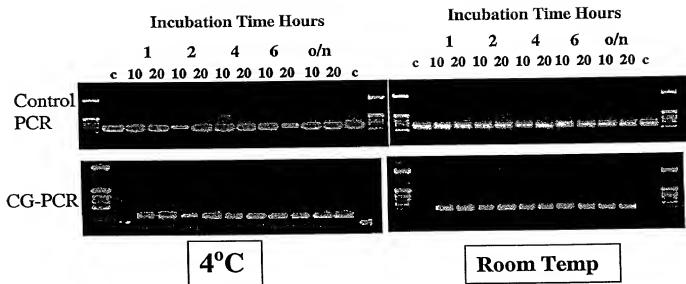
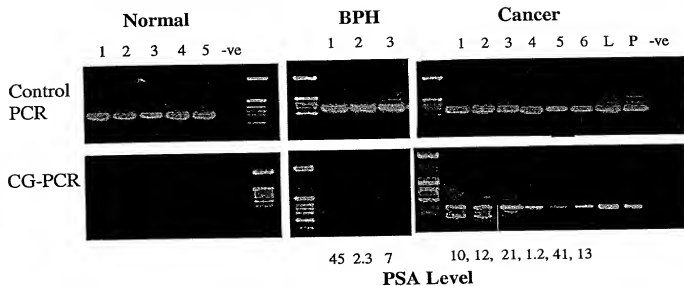


Figure 7B



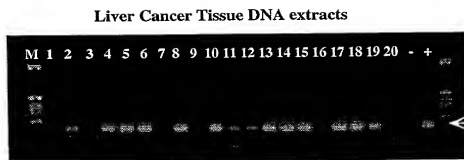
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Figure 8



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Figure 9



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Figure 10

Test Oligo's

Unconverted PCR probe		
Converted Bases of 10	10 Cancer Samples	BPH Samples
2	10	1
8	10	2
8	1	3
1	2	4
8	3	
0	4	

Converted PCR probe		
Converted Bases of 10	10 Cancer Samples	BPH Samples
2	10	1
8	10	2
8	1	3
1	2	4
8	3	
0	4	

Control Oligo

Converted PCR probe		
Converted Bases of 10	10 Cancer Samples	BPH Samples
2	10	1
8	10	2
8	1	3
1	2	4
8	3	
0	4	

DECLARATION AND POWER OF ATTORNEY

As the below named inventors, we hereby declare that our residence, post office address and citizenship are as stated below next to our names; that we verily believe we are the original and joint inventors of the subject matter claimed in the application for which a patent is sought in the application entitled:

ASSAY FOR METHYLATION IN THE GST-PI GENE

which application is:

☐ the attached application

(for original application)

☒ Application No. 09/673,448

filed October 16, 2000, and amended on

October 16, 2000

(for declaration not accompanying application)

that we have reviewed and understand the contents of the specification of the above-identified application, including the claims, as amended by any amendment referred to above; that we acknowledge our duty to disclose information of which we are aware which is material to the patentability of this application under 37 C.F.R. § 1.56, that we hereby claim priority benefits under Title 35, United States Code §§ 119, § 112 or § 365 of any provisional application or foreign application(s) for patent or inventor's certificate listed below and have also identified on said list any foreign application for patent or inventor's certificate on this invention having a filing date before that of any foreign application on which priority is claimed:

Application Number	Country	Filing Date	Priority Claimed
PCT/AU99/00306 PP 3129	PCT Australia	April 23, 1999 April 23, 1998	Yes Yes

We hereby claim the benefit of Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the material matter of each of the claims of this application is not disclosed in a United prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, We acknowledge our duty to disclose any information material to the patentability of this application under 37 C.F.R. 1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application No.	Filing Date	Status
-----------------	-------------	--------

We hereby appoint John H. Mison, Reg. No. 18,879; Thomas J. Macpeak, Reg. No. 19,292; Robert J. Seas, Jr., Reg. No. 21,092; Darryl Mezin, Reg. No. 22,063; Robert V. Sloan, Reg. No. 22,775; Peter D. Olcay, Reg. No. 24,513; I. Frank Ochoa, Reg. No. 24,625; Waddell A. Biggart, Reg. No. 24,861; Louis Gubinsky, Reg. No. 24,835; Neil B. Slagel, Reg. No. 25,200; David I. Cushing, Reg. No. 28,703; John E. Inge, Reg. No. 26,916; Joseph J. Kuch, Jr., Reg. No. 26,577; Sheldon I. Landerman, Reg. No. 25,420; Richard C. Turner, Reg. No. 29,710; Howard L. Bornstein, Reg. No. 26,665; Alan J. Kaspar, Reg. No. 25,424; Kenneth J. Buchheit, Reg. No. 31,833; Gordon Kit, Reg. No. 30,764; Susan J. Mack, Reg. No. 30,951; Frank L. Bornstein, Reg. No. 31,484; Mark Boland, Reg. No. 32,137; William H. Mendir, Reg. No. 32,156; Scott M. Daniels, Reg. No. 32,562; Brian W. Hannen, Reg. No. 22,278; Abraham J. Rosner, Reg. No. 33,276; Bruce E. Kramer, Reg. No. 33,725; Paul E. Nells, Reg. No. 33,102; Brent S. Sylvester, Reg. No. 32,765; Robert M. Masiera, Reg. No. 35,603 and George E. Lehnigk, Reg. No. 36,559, our attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith, and request that all correspondence about the application be addressed to **SUGHERUE, MISON, ZINN, MACPEAK & SEAS, PLLC**, 2100 Pennsylvania Avenue, N.W., Washington, D.C. 20037-3213.

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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City		State/Country		

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Citizenship Australian

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Citizenship Australian

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Residence _____ Signature _____
City State/Country First Name Middle Initial Last Name

Post Office Address: _____

Citizenship _____

Date _____ Fifth Inventor _____
Residence _____ Signature _____
City State/Country First Name Middle Initial Last Name

Post Office Address: _____

Citizenship _____

Date _____ Sixth Inventor _____
Residence _____ Signature _____
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Post Office Address: _____

Citizenship _____

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MILLER, Douglas S.
MOLLOY, Peter L.

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